Expression of human glutamic acid decarboxylase, a major autoantigen in Type 1 diabetes, in transgenic plants

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Abstract

The smaller isoform of the enzyme glutamic acid decarboxylase (GAD65) is a major islet autoantigen in autoimmune Type 1 diabetes mellitus (T1DM). Transgenic plants expressing human GAD65 (hGAD65) are a potential means of direct oral administration of the islet autoantigen in order to induce oral tolerance and prevent clinical onset of disease. We reported the successful generation of transgenic tobacco and carrot that express immunoreactive full-length hGAD65. However, the expression levels of the recombinant protein (0.04% of total soluble protein) were inadequate to plan oral tolerance induction studies in animal models. To enhance the expression levels of hGAD65 in transgenic plants, we have engineered the molecule for targeting the cytosol, which resulted in a 5-fold increase of hGAD65 expression levels. We also investigated transient expression of hGAD65 in plants. Transient expression of wild-type, full-length hGAD65 in N. benthamiana mediated by PVX infection was associated with expression levels of immunoreactive protein as high as 2.2% of total soluble protein. This substantial improvement of the expression of hGAD65 in plants paves the way for immunoprevention studies of oral administration of GAD65-containing transgenic plant material in animal models of spontaneous autoimmune diabetes.

Introduction

Type 1 (insulin-dependent) diabetes mellitus (T1DM) is one of the most common chronic diseases of childhood, affecting 0.3-0.4% of the general population (Atkin-
son and Maclaren 1994) and is caused by the autoimmune destruction of insulin-secreting pancreatic beta cells. The disease results from the interplay between a pre-disposing complex genetic background and still incompletely defined environmental factors (Lernmark and Falorni 1997). A major genetic contribution is provided by the polymorphism of HLA class II genes, located on the short arm of chromosome 6. In particular, while HLA-DRB1*03-DQA1*0501-DQB1*0201 and DRB1*04-DQA1*0301-DQB1*0302 are positively associated with genetic risk for T1DM, DRB1*1501-DQA1*0102-DQB1*0602 is negatively associated (Sanjeevi et al. 1995). Other major histocompatibility complex genes, such as HLA-DPB1 (Noble et al. 2000), MICA (Gambelunghe et al. 2001) and HLA-B (Nejentsev et al. 1997) also modulate the genetic risk conferred by HLA-DR and -DQ haplotypes. Although 45-50% of the genetic risk can be attributed to HLA class II genes, other non-HLA regions are also linked and associated with genetic risk, namely the CTLA-4 gene (on chromosome 2; Nisticò et al. 1996) and a microsatellite in the 5′ flanking region of the insulin gene (on chromosome 11; Owerbach and Gabbay 1993).

The islet autoimmune response is evidenced by the appearance of circulating autoantibodies directed against a long series of islet autoantigens, among which the most relevant are the smaller isoform of the enzyme glutamic acid decarboxylase (GAD65), insulin and tyrosine-phosphatase-like IA-2/IA-2β/phogrin antigens (Falorni and Calcinaro 2003). GAD65 autoantibodies (GADA) and IA-2 autoantibodies (IA-2A) comprise most (although not all) of the antibody specificity of islet-cell antibodies (ICA), detected by indirect immunofluorescence on cryostatic sections of human pancreas. Over 95% of T1DM children are positive for at least one of the four major autoantibody specificities (GADA, IA-2A, insulin autoantibodies - IAA - and ICA; Falorni and Calcinaro 2003). More precisely, around 60-80% of newly diagnosed T1DM subjects are positive for GADA, irrespective of age at disease onset (Vandewalle et al. 1995). Conversely, IA-2A and IAA are predominantly detected in T1DM children, while they are infrequent in adult-onset T1DM (Vandewalle et al. 1995; Gorus et al. 1997). IA-2A and IAA tend to occur more frequently in subjects with rapid beta cell destruction and their production appears to be the secondary result of a massive release of islet autoantigens from the damaged beta cells. GADA production is a more sensitive marker of chronic islet autoimmunity and represents the most important marker of adult-onset T1DM. They also identify the so-called latent autoimmune diabetes in adults (LADA) in diabetic subjects who are non-insulin-dependent at diagnosis, but who rapidly progress to insulin deficiency within a few years (Falorni et al. 2000). The analysis of the variable chain gene usage and the degree of somatic mutations needed for the formation of human GAD65 monoclonal autoantibodies (GAD65 MoAb; Richter et al. 1995) demonstrated a non-random ratio of replacement compared to silent mutations in the complementary determining regions (CDR) of four of seven studied GAD65 MoAb. The same study showed a high relative avidity for the autoantigen, suggesting that the investigated GAD65 MoAb were produced by B-lymphocytes
with an efficient help from disease-related T-lymphocytes and following several rounds of reactivation and selection. This, in turn, generated autoantibodies at increasing affinity that developed as a result of an autoantigen-driven mechanism and not as a consequence of a secondary effect of β-cell destruction.

A complicating factor is the existence of at least another GAD isoenzyme of molecular weight 67,000 (GAD67; Bu et al. 1992). GAD65 and GAD67 are encoded by two different genes located on chromosome 10 (Karlsen et al. 1991; Bu et al. 1992) and chromosome 2 (Michelsen et al. 1991; Bu et al. 1992), respectively. Both GAD65 and GAD67 are expressed in the central nervous system in humans and other animals (Petersen et al. 1993). In human islets, however, only GAD65 is expressed at high levels, in contrast to rat islets where both isoenzymes are expressed (Petersen et al. 1993). The subcellular localization also differs between GAD65 and GAD67, with the former predominantly hydrophobic and anchored to synaptic-like vesicles, and the latter with a preferential peri-nuclear (i.e. cytoplasmic) localization (Solimena et al. 1993). The different subcellular localization between GAD65 and GAD67 has been shown to depend on signals located on the NH$_2$-terminal domains of the two isoenzymes (Solimena et al. 1994). Autoantibodies cross-reacting with both GAD65 and GAD67 can be detected in approximately 15% of T1DM children, but they can be displaced by an excess of human GAD65 in almost all cases, thus demonstrating that GAD67 is not an autoantigen in human T1DM (Falorni et al. 1994) and that GAD67 antibodies represent only a subtype of GAD65 antibodies directed to epitope(s) of GAD65 that share similarities with homologous domains of GAD67 (Falorni et al. 1996).

Although islet autoantibodies are the gold immunological parameter to demonstrate an ongoing islet autoimmune process and are routinely used in clinical practice, the islet beta cell destruction is a consequence of a T-cell mediated process, and autoantibodies seem to play little or no pathogenic role. Indeed, the possibility of transferring the disease as a direct consequence of bone marrow transplantation (Lampeter et al. 1993) and the recent demonstration of the development of T1DM in a male subject with severe B-cell deficiency and X-linked agammaglobulinemia (Martin et al. 2001) imply that neither B-lymphocyte function nor islet autoantibodies are critically involved in the pathogenesis of T1DM.

Autoreactive T-cells against GAD65, insulin and other autoantigens have been detected in T1DM patients and pre-diabetic subjects (Shehadeh and Lafferty 1993; Tree and Peakman 2004). The possibility of prolonging the clinical remission of the disease by using immunosuppressive drugs, such as cyclosporine (Feutren et al. 1986) or an anti-CD3 monoclonal antibody (Herold et al. 2002) confirms the central role of cell immunity in the pathogenesis of T1DM in humans. Furthermore, several studies indicate the presence of multiple abnormalities of NK cells, CD45R subpopulations, dendritic cells and CD4+ and CD8+ T-cells in T1DM patients (Roep 2003). The causes of such abnormalities and their relevance in the natural history of the disease remain unclear, but they might suggest that an immunoregul-
latory defect may be responsible for the progression of the islet autoimmune process towards clinical diabetes. Thus, several studies indicate the existence of a regulation dysfunction in the genesis of T1DM. Indeed, autoreactive T cells (as well as islet autoantibodies) can be detected in several non-diabetic subjects, which demonstrates the existence of two different types of islet autoimmunity: destructive and not destructive.

Insulitis, i.e. the morphological expression of islet autoimmunity, is caused by infiltration of pancreatic islets by chronic inflammatory cells (both CD8+, but also CD4+ T cells). A similar histological finding is common in all endocrine autoimmune diseases, such as autoimmune thyroiditis, autoimmune adrenal insufficiency and autoimmune premature ovarian failure. However, the infiltration of a target organ by autoreactive T cells is not necessarily the expression of clinical disease as only a fraction of subjects with an infiltrating organ-specific autoimmune process presents an extensive cell destruction that causes clinical signs and symptoms. Thus, a predisposing genetic background appears to be critical for the development of islet autoimmunity, but the progression towards clinical T1DM in subjects with autoantibodies and autoreactive T cells is under the control of regulatory cells and environmental factors (Ziegler et al. 2003) that may act as modulators of the autoimmune process.

The heterogeneous genetic background, different environmental factors and the unavailability of pancreatic tissue for histology studies limit the possibility of directly studying the pathogenesis of T1DM in humans. The non-obese diabetic (NOD) mouse (Makino et al. 1980) and the BB rat (Rossini et al. 1979) are inbred rodent strains which develop spontaneous insulin-dependent diabetes. The autoimmune diabetes seen in these animal models is remarkably similar to the human disease. Studies in the NOD mouse have confirmed the importance of different T-helper subtypes in the pathogenesis of T1DM. Thus, T1DM appears to be the result of predominant Th1-like responses, while the stimulation of Th2-like clones can modify the natural history of the disease, thus preventing the occurrence of clinical diabetes (Mosmann et al. 1986; Mosmann and Coffman 1989). In NOD mice, stimulation of the immune system by adjuvant therapy results in the switch of the autoimmune response from a destructive to a non-destructive type with a preferential expansion of Th2 cells in the insulitis (Shehadeh et al. 1994). Other immunoprevention studies indicate that Th2-like, anti-inflammatory cytokines, such as IL-10 and IL-4 play a synergistic role in the protection from autoimmune diabetes (Calcinaro et al. 1997).

T1DM is a major health problem. The need for life-long insulin therapy, the constant risk of developing acute complications such as ketoacidosis or hypoglycaemia, the seriousness and frequency of chronic complications provide a strong rationale for the development of efficient, safe and low-cost strategy of immunoprevention of this disease. Several immunoprevention strategies for secondary prevention of T1DM in pre-diabetic subjects with immunological signs of an ongoing
autoimmune process have been tested, including general immunosuppression (Stiller et al. 1984), the use of radical scavengers (Vague et al. 1987), the use of nicotinamide (Gale et al. 2004) the induction of β-cell rest by therapeutic administration of insulin (Keller et al. 1993; Diabetes Prevention Trial 2002) and immunomodulation with adjuvants (Shehadeh et al. 1994). First-degree relatives of diabetic patients are an ideal population for these studies of secondary prevention of T1DM. However, based on the fact that at least 90% of T1DM subjects have no affected relatives, primary prevention of T1DM in the general population is the ultimate goal. The discouraging results of clinical studies of secondary prevention have generated a fundamental dilemma: treating subjects at high risk for T1DM with an ongoing autoimmune process might hamper the efficacy of the treatment. At a late stage of the disease, only aggressive immunosuppressive treatments may be effective, but at the cost of a high risk of dangerous side effects (such as increased risk for malignancies). On the other hand, an early treatment (in genetically predisposed individuals, with no detectable markers of islet autoimmunity) would imply the study of a large number of subjects who will never develop the disease. In this latter approach, safety of the treatment is a critical issue. No one of the therapies so far investigated combines all the features, i.e. efficacy, safety, specificity, low cost and applicability to the general population, which are required for primary prevention. An attractive strategy would be immunomodulation by induction of oral tolerance with islet autoantigens. This approach would theoretically satisfy all the needed requirements with the exception of the present high cost of producing large quantities of recombinant human proteins.

GAD65 is a major autoantigen associated with autoimmune diabetes and a key molecule in the disease process (Yoon et al. 1999), and the parenteral administration of GAD65 has proven effective in preventing the disease in animal models of the disease (Kaufman et al. 1993; Tish et al. 1993). Accordingly, studies of oral tolerization with hGAD65 have a strong rationale.

Oral tolerance and T1DM prevention

Oral tolerance consists of the oral administration of antigens that could alter the response of the immune system. This is a form of peripheral immune tolerance in which mature lymphocytes in the peripheral lymphoid tissues are rendered non functional or hypo-responsive by prior oral administration of an antigen (Toussirot 2002). Induction of oral tolerance has been reported to modify the natural history of several autoimmune diseases, both in experimental models and in pilot human trials (Weiner et al. 1994; Moling and Mian 2003). This therapeutic approach requires the oral administration of antigens and the active participation of the gut-associated lymphoid tissue (GALT), a tissue comprising Peyer’s patches, intraepithelial cells and villi containing epithelial cells.
The three principal immunological mechanisms by which orally administered antigen induce tolerance are clonal deletion, anergy and antigen-driven suppression (Strobel 2002). The primary factor determining which form of tolerance will be developed after oral administration of antigen is the antigen dosage. Thus, it is thought that low doses of antigen induce the generation of active suppression via regulatory T cells in the GALT, which then migrate to the systemic immune system. These regulatory T cells produce down-regulatory cytokines such as IL-4, IL-10 and TGFβ, a Th2-like cytokine pattern. Conversely, high doses of antigens favour anergy or clonal deletion. The phenomenon in which regulatory cells, as generated by oral tolerization, are primed in an antigen-specific manner, but act in the respective microenvironment in a non-antigen-specific manner, is called bystander suppression.

Studies in NOD mice have shown that parenteral and nasal administration of GAD65 or GAD65-peptides can prevent or delay the onset of the disease (Kaufman et al. 1993; Tish et al. 1993; Tian et al. 1996). Moreover, immunological tolerance can also be induced in NOD mice by oral administration of disease-associated autoantigens (Zhang et al. 1991), suggesting that the clinical onset of T1DM might be prevented by oral prophylaxis with GAD65.

During early lymphocyte accumulation and infiltration in mice, the mucosal addressin cell adhesion molecule (MAdCAM-1) becomes expressed on islet vessels of NOD and preferentially mediates the homing of β7-integrinhigh, L-selectine-positive mucosal lymphocytes (Hanninen et al. 1993; Yang, et al. 1994; Hänninen et al. 1996). Therefore, MAdCAM-1 islet expression could favor early accumulation of mucosal associated lymphocytes in pancreatic islets. In addition, α4β7-integrin-positive, GAD-specific, circulating lymphocytes have been demonstrated in T1DM patients (Paronen et al. 1997). Hence, GALT may play a critical role in favoring islet-specific autoimmunity in diabetes-prone individuals, even in humans. Supporting evidence to this theory come from recent observations, both in humans and in animal models, that have drawn attention to a possible involvement of the gastrointestinal tract in the pathogenesis of autoimmune diabetes, in the light of the reported association of some dietary antigens, such as cow’s milk proteins, gliadin or other cereal components, and of enteric infections with the disease (Holmes 2001; Akerblom et al. 2002; Vaarala 2002; Norris et al. 2003). Furthermore, a link between the GALT and the development of autoimmune diabetes has been already shown in animal models: indeed mesenteric lymphocytes from 3-week-old NOD mice had a high diabetogenic potential (Jaakkola et al. 2003), and diet manipulations modified disease incidence as well as the pattern of islet infiltrating lymphocytes and of cytokine production (Scott et al. 1997). Proper immunomodulation of GALT by disease-related antigens may generate or stimulate regulatory cells whose recirculation to the site of islet infiltration would produce locally anti-inflammatory cytokines with subsequent modification of the type of insulitis and prevention of clinical diabetes.
However, induction of oral tolerance in mouse requires the prolonged administration of autoantigens in the range of milligrams/week (Zhang et al. 1991). Poor GAD65 protein solubility in bacteria and inadequate production from eukaryotic cells have so far precluded the use of this approach for the large-scale production of GAD65 for oral tolerance studies. The problem could be overcome by the use of transgenic plants that express high levels of hGAD65 and could be the source for oral administration of the autoantigen. Studies in NOD mice indicate that the incidence of T1DM could be reduced by feeding the animals with transgenic plants that express cholera toxin B subunit-insulin conjugates (CTB-insulin; Arakawa et al. 1998) or mouse GAD67 (Ma et al. 1997). However, the low number of animals studied in the latter study (Ma et al. 1997) with mouse GAD67, the inadequate quantification of the expression levels of the antigen in transgenic plants that likely resulted in a gross overestimation, and the limited immunological role of GAD67 in human T1DM, make it difficult to decipher the actual relevance of the use of plant-expressed mouse GAD67. In view of the fact that GAD65 is the major autoantigen associated with human chronic islet autoimmunity, the construction and characterization of transgenic plants that express hGAD65 is a logical goal. Because the two major isoforms of plant GAD so far isolated have an amino acid identity with human GAD around 17% (Zik et al. 1998), they are not recognized by human T1DM human antibodies. Therefore, human GAD65 expressed in plant organs by transgenic approach can be unambiguously quantified by fluid-phase radioimmunoassay (RIA; Falorni et al. 1995) with serum from a T1DM patient as a source of anti-GAD65 antibodies.

Transgenic plants expressing hGAD65

We reported the first demonstration of the construction of transgenic plants expressing human, full-length GAD65 (Porceddu et al. 1999). We used Agrobacterium carrying the plasmid pBIN35SGAD65 containing human GAD65 cDNA under the control of the constitutive Cauliflower Mosaic Virus (CaMV) promoter 35S to transform tobacco leaf discs and carrot hypocotyls (Figure 1A). The Northern blot analysis with a radiolabelled GAD65 probe demonstrated that the tobacco transformed plants produce a transcript of the correct length (Figure 1B). Biochemical characterisation of proteins was carried out on the two tobacco lines with the highest accumulation levels (Figure 1C).
In animal cells, human GAD65 is membrane-anchored by signal located in the NH$_2$-terminal region (Solimena et al. 1994). The association of GAD65 with membranes in human cells is mediated by way of protein-protein interactions, with accessory effects contributed by palmitoylation (Namchuk et al. 1997). To characterise sub-cellular localization of human GAD 65 in plant cells, we performed immunogold labelling and electron microscopy of transgenic tobacco leaf tissue. This analysis showed that gold particles were almost exclusively localized to chloroplast tylacoids and mitochondria, with a very reduced labelling of the cytoplasm (Figure 1D). These data indicate that human GAD65 is mainly targeted to plant organelle membranes, thus supporting the existence of a targeting system similar to that observed in animal cells.
To test whether the GAD65 produced in transgenic tobacco preserves conformational autoantibody epitopes, we took advantage of a fluid-phase RIA with serum from a T1DM patient as a source of anti-GAD65 antibodies. Human GAD65 in leaf extracts from the transgenic tobacco plants were immunoprecipitated by T1DM autoantibodies, thereby providing evidence that conformational epitopes of plant-expressed GAD65 were conserved. In addition, scalar dilution of a standard rhGAD65 allowed hGAD65 to be quantified in transgenic tobacco leaves. Mean concentrations ranged between 297 and 826 ng/g of leaf, which corresponds to 0.014-0.040% of total soluble plant proteins. Human GAD65 expression levels in transgenic carrot taproots were 0.009-0.012% of total soluble proteins. The enzymatic activity of plant-expressed human GAD65 was tested in an immunoenzymatic assay. The assay was performed on transgenic tobacco tissue and it demonstrated that plant-expressed hGAD65 retains its enzymatic activity. No morphological or developmental alteration in any of the human-GAD65 expressing primary transgenic tobacco plants grown to maturity under green-house conditions was observed.

Although the hGAD65 expression levels obtained in this experiment were similar to those reported for other human proteins expressed in plants such as hemoglobin (Dieryck et al. 1997), alpha-lactalbumin (Takase and Hagiwara 1998), and beta casein (Chong et al. 1997), they were considered insufficient for oral administration of transgenic plant material in pre-diabetic animals because they

Figure 1D. Sub-cellular localization of in planta-expressed human GAD65. Electron microscope photograph of leaf tissues of transgenic tobacco where chloroplasts and mitochondria were immunogold labeled using a GAD65-specific antiserum.
could enable the administration of only a few micrograms of autoantigen per mouse per week, a dose 100- to 200-fold lower than that required, as calculated from the results observed with purified, recombinant human insulin. Not surprisingly, a recent study showing similar in planta-expression levels of human GAD65 (Ma et al. 2004) failed to detect any significant positive effect of oral administration of GAD65 and only the co-administration of GAD65 and IL-4 (a potent anti-inflammatory cytokine that was already shown to be able to prevent the development of autoimmune diabetes in the NOD mouse) reduced the incidence of diabetes in NOD mice. Unfortunately, this study (Ma et al. 2004) does not provide any useful novel information on the possibility of inducing oral tolerance by administration of in planta-expressed human GAD65 since it was based on the use of autoantigen doses which were too low to be effective.

**Improvement of hGAD65 stable expression**

With the aim of properly testing the possibility of inducing oral tolerance by oral administration of adequate doses of in planta-expressed human GAD65, we are currently addressing the problem of increasing the expression levels of the autoantigen in tobacco plants. We recently reported (Avesani et al. 2003) the development of an expression cassette that increased the stable expression of hGAD65 in plants by maintaining the molecule in the cytosol. In a previous study, cytosolic expression levels of some heterologous proteins were higher than those of membrane-bound proteins in transgenic plants (Mason et al. 1996). A chimeric protein was obtained by substituting the NH2-terminal region of hGAD65 with the corresponding region of GAD67. As already discussed, GAD67 is not an autoantigen in T1DM (Falorni et al. 1996) and lacks membrane-anchoring signals in the NH2-terminal domain and is thus located in the cytosol.

A GAD67/65 chimeric cDNA in which the first 87 amino acids of human GAD65 are substituted with a homologous region of rat GAD67 was constructed by overlapping PCR. The resulting expression vector pBIN35SGAD67/65 contained the chimeric GAD67/65 cDNA cloned between the Cauliflower Mosaic Virus 35S (CaMV35S) promoter and the Agrobacterium tumefaciens Nopaline synthase (NOS) terminator (Figure 2A). The GAD67/65 expression vector pBIN35SGAD67/65 and the control vector pBIN19 were transformed into two different tobacco cultivars (Petit Havana SR1 and Lonibow) via Agrobacterium-mediated gene transfer. Overall, 145 SR1 and 81 Lonibow tobacco plants were regenerated and analyzed by PCR for the presence of the GAD67/65 transgene. In total, 202 regenerants had integrated the GAD67/65 cDNA in their genome.
Transgenic tobacco plants were subjected to RT-PCR analysis using one primer for the GAD67 portion and the other for the GAD65 sequence in order to detect the GAD67/65-specific transcript (Figure 2B). Only plants expressing the chimeric protein showed a specific PCR fragment.

Total extracts of soluble protein from transgenic plant leaves expressing GAD67/65-specific transcript and control lines were subjected to western blot analysis. In the transgenic plants, a GAD65-specific signal of the correct length was detected, compared to that generated by the full-length rhGAD65 produced in the baculovirus-insect cell expression system (Figure 2C).

**Figure 2A.** Schematic structure of the T-DNA region of binary vector pBin35SGAD67/65 for expression of chimeric GAD67/65. The expression cassettes for GAD67/65 and neomycin phosphotransferase (NPTII, to confer kanamycin resistance to plants) are located between the right and left T-DNA borders (RB, LB). *Agrobacterium*-mediated transfer of the T-DNA into plant cells directs its insertion into the plant nuclear chromosomal DNA. Transcription of GAD67/65 is driven by cauliflower mosaic virus 35S promoter (CaMV 35S) while the *Agrobacterium tumefaciens* Nopaline synthase terminator (NOS t) mediates 3end processing of the transcript.

**Figure 2B.** RT-PCR analysis of cDNA from total RNA extracted from mature leaves of transformed mature leaves. Isolated cDNA was subject to PCR using the same primers used for PCR analysis. Lane 1: molecular size marker (Gene Ruler DNA Ladder mix, MBI Fermentas). Lanes 2-6: clones 20, 27, 43, 61, 55. Lane 7: GAD65-transformed plant. Lane 8: untransformed plant: Lane 9: transgenic clone 20 whose RNA was not reverse transcribed.

**Figure 2C.** Western blot analysis of total proteins. In each lane, 20 µg of extracted protein were loaded. As positive control (lane 1), 15 ng of purified recombinant human GAD65 was loaded. Lane 2-4: clones 20, 27, 43 respectively. Lane 5: GAD65-transformed plant. Lane 6: negative control (no-transformed plant).
Immunogold labelling and electron microscopy of leaf tissue indicated that the chimeric GAD67/65 is mainly localized in the plant cell cytosol of the transgenic plants (Figure 2D).

Leaf extracts from all transformed plants were subjected to RIA analysis to quantify the level of immunoreactive protein. In transgenic leaves, the GAD67/65 protein was estimated to represent an average of $0.04 \pm 0.01\%$ of total soluble plant proteins. The transgenic plant having the highest level of expression contained $0.19\%$ of GAD67/65 of total soluble plant proteins. These data indicate that the highest stable expression of the recombinant GAD67/65 protein ($0.19\%$ of total soluble plant proteins) in transgenic plants is 5-fold higher than the level achieved for hGAD65 ($0.04\%$ of total soluble plant proteins).

To test whether the enhanced accumulation of GAD67/65 vs. GAD65 proteins correlated with transcript accumulation, we performed RT-PCR. We compared the relative quantity of hGAD65 transcript in the highest expressing GAD65 transgenic T2 plant ($0.04\%$ of total soluble plant proteins) with the one of GAD67/65 in the highest expressing GAD67/65 transgenic T2 plant ($0.19\%$ of total soluble plant proteins). No significant difference was found in the relative quantity of the GAD65 transcript in the two transgenic plants. Therefore, we concluded that the observed difference in protein expression is likely not due to a difference in the levels of transcript accumulation. We propose that hGAD65 protein in the cytosol is dramatically more stable than the hGAD65 targeted to the membrane. A diverse translation efficiency may also be possible in the two different transgenic plants.
Transient expression of hGAD65

We also reported (Avesani et al. 2003) the use of a potato virus X (PVX)-based vector transiently expressing wild-type, full-length hGAD65 (PVX.GAD65). In vitro RNA transcripts of PVX:GAD65 were used to inoculate Nicotiana benthamiana plants. Twelve days later, leaves from plants showing systemic infection were analysed by western blotting; this analysis demonstrated that infected leaves expressed a recombinant protein of the expected size (Figure 3).

The recombinant hGAD65 transiently expressed in *N. benthamiana* infected plants was successively quantified by RIA. These plants expressed hGAD65 at an average level of 2.16% of total soluble plant proteins. Hence, the level of expression obtained transiently in *N. benthamiana* PVX.GAD65 infected leaves is more than 10-fold higher than the highest level of stable expression (0.19% of total soluble plant proteins). Moreover, there was very little variation in the level of expression of hGAD65 among the plants analysed, indicating that transient expression is highly reliable and is not affected by endogenous and/or environmental factors. This result confirms the ability of plant virus-based transient expression to direct rapid and high-level expression of foreign genes in mature, differentiated plant tissue and demonstrates the enormous advantage of plant viral-based transient expression in comparison to stable transformation strategies. The high level to which many viruses and virus-encoded proteins accumulate, the avoidance of costly fermentation systems and the flexibility in scale of production afforded by plants are potential advantages of virus-mediated protein production in plants (Lomonossoff 2001).

Conclusions

In conclusion, our results show that it is feasible to use transgenic technology to produce biologically active human GAD65 *in planta*. In particular, we demonstrated that targeting the major human islet autoantigen GAD65 to the cytosol of plant cells leads to a significant increase in its expression level in transgenic plants. In *N. benthamiana*, transient expression of hGAD65 mediated by infection with the PVX vector determined a further dramatic increase in recombinant expression.
levels. The expression levels of transgenic plants containing GAD67/65 and hGAD65 and the level of recombinant protein hGAD65 achieved transiently in *N. benthamiana* infected leaves is shown in Figure 4. These data indicate that the highest stable expression of the recombinant GAD67/65 protein (0.19% of total soluble plant proteins) is five-fold higher than the highest level achieved for hGAD65 (0.04% of total soluble proteins). However, this value is still 10-fold lower than the highest level of expression obtained transiently in *N. benthamiana* PVX.GAD65 infected leaves (2.25% of total soluble plant proteins).

The improvement in the expression of hGAD65 herein reported in plants paves the way for immunoprevention studies of oral administration of GAD65-containing transgenic plant material in animal models of spontaneous autoimmune diabetes.
References


Michelsen BK, Petersen JS, Boel E, Modrrup A, Dyberg T, Madsen OD (1991) Cloning, characterization and autoimmune recognition of rat islet glutamic acid decarboxylase in insulin-depen-
Namchuk M, Lindsay L, Tusck CW, Kanaani J, Baeckkeskov S (1997) Phosphorylation of serine residues 3, 6, 10 and 13 distinguishes membrane anchored from soluble glutamic acid decarboxylase 65 and is restricted to glutamic acid decarboxylase 65alpha. J Biol Chem 272:1548-1557
Scott FW, Cloutier HE, Kleemann R, Woerz-Pagensterr U, Rowell P, Modler HW, Kolb H (1997) Potential mechanisms by which certain foods promote or inhibit the development of spontaneous diabetes in BB rats: dose, timing, early effect on islet area, and switch in infiltrate from Th1 to Th2. Proc Natl Acad Sci USA 88:8754-8758
Th2 cells. Diabetes 46:589-598