Engineering Secreted Proteins for Gene Transfer and DNA Vaccination

JingXue Liu

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ENGINEERING SECRETED PROTEINS FOR
GENE TRANSFER AND DNA VACCINATION

by

JingXue Liu

A Dissertation Submitted in Partial Fulfillment
of the Requirements for the Degree of Doctor of
Philosophy in Microbiology and Molecular Genetics

June 1999
Each person whose signature appears below certifies that this dissertation in their opinion is adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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<td>aa</td>
<td>amino acid(s)</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>ADA</td>
<td>adenosine deaminase</td>
</tr>
<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>carboxyl terminus</td>
</tr>
<tr>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>CAT</td>
<td>chloramphenicol acetyl transferase</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified (minimal) Eagle's (essential) medium</td>
</tr>
<tr>
<td>ds</td>
<td>double stranded</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>GAD65</td>
<td>glutamic acid decarboxylase 65</td>
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<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>IDDM</td>
<td>insulin-dependent diabetes mellitus</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>IL2SP</td>
<td>interleukin-2 signal peptide</td>
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<tr>
<td>IM</td>
<td>intramuscular</td>
</tr>
<tr>
<td>INF</td>
<td>interferon</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>LB</td>
<td>Luria-Bertani medium</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>MAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MuLV</td>
<td>murine leukemia virus</td>
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<tr>
<td>MW</td>
<td>molecular weight</td>
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<tr>
<td>N</td>
<td>amino terminus</td>
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<tr>
<td>NOD</td>
<td>non-obese diabetic</td>
</tr>
<tr>
<td>OD&lt;sub&gt;260&lt;/sub&gt;</td>
<td>optical density at 260 nm</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
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<tr>
<td>RLU</td>
<td>relative light units</td>
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<tr>
<td>RUC</td>
<td>Renilla reniformis luciferase</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>SE</td>
<td>standard error</td>
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<tr>
<td>SEAP</td>
<td>secreted alkaline phosphate</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SGAD55</td>
<td>secreted GAD 55</td>
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<tr>
<td>SRUC</td>
<td>secreted Renilla reniformis luciferase</td>
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<tr>
<td>SV40</td>
<td>simian virus 40</td>
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<tr>
<td>TEMED</td>
<td>N, N, N', N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TH1/TH2</td>
<td>T helper cell subset one/two</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>wt/vol</td>
<td>weight per volume</td>
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<td>wt/wt</td>
<td>weight ratio</td>
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ABSTRACT

ENGINEERING PROTEINS FOR SECRETION:
APPLICATION TO GENE TRANSFER AND DNA VACCINATION

by

Jingxue Liu

In recent years gene therapy has become a promising way of alleviating incurable human ailments, its concept emerging as the ultimate therapy for many infectious and genetic diseases. Two important aspects of the development of successful gene therapy protocols are the ability to monitor gene transfer readily, and the establishment of new protocols for treating specific diseases. In this work, *Renilla* luciferase and human glutamic acid decarboxylase (GAD) 65 were engineered for secretion to address some aspects of these issues.

Secreted reporter proteins are promising tools to study gene transfer and expression in a non-destructive manner, and bioluminescent proteins are particularly convenient to use for that purpose. To generate a secreted bioluminescent marker protein, secreted *Renilla* luciferase (SRUC), was engineered by fusing the human interleukin-2 signal peptide to *Renilla* luciferase. We further modified the *Renilla* luciferase gene using site-directed-mutagenesis to obtain a mutant form of *Renilla* luciferase, SRUC3, with dramatically improved stability. SRUC3 provides a rapid,
sensitive, and inexpensive assay that does not require disruption of transfected cells. Data from animal experiments suggested that SRUC3 has the potential to be used as an in vivo marker protein. Although SRUC3 activity was not detected from animal blood, data indicated that improvement of the assay after further engineering of the sruc3 gene and SRUC3 protein could allow detection of Renilla luciferase activity in the blood and serum in the future.

To evaluate the potential of genes encoding engineered secreted autoantigens for the treatment of autoimmune disease using gene vaccination, human GAD65 was engineered to be secreted by mammalian cells. GAD65 is a major autoimmune antigen involved in the development of type I diabetes in both non-obese diabetic (NOD) mice and human patients. After the removal of the N-terminal end of GAD65, the truncated GAD fragment was fused to the C-terminal of the human interleukin-2 leader peptide, and a secreted form of GAD (SGAD55) was obtained. The intramuscular injection of plasmid DNA encoding GAD65 and SGAD55 into three-week-old NOD mice showed a dramatic reduction of development of insulitis, the first symptom of diabetes in NOD mice.
CHAPTER ONE
INTRODUCTION

A. Naturally and artificially secreted proteins

Protein secretion is one of the most intricate cellular events in eukaryotic cells. The internal membrane system provides a means whereby eukaryotic cells can regulate the delivery of newly synthesised proteins to the outside of cells. The protein secretion pathway includes several cellular compartments, such as the endoplasmic reticulum (ER) and Golgi apparatus. Newly synthesised proteins enter the secretion pathway in the ER by crossing the ER membrane from the cytosol, and are subsequently transported from the ER to Golgi apparatus, and then to the cell surface. Transfer vesicles, which transfer proteins from membrane to membrane or from lumen to lumen (or to extracellular space) by cycles of vesicle budding and fusion, mediate the secretion process. The fusion of the vesicles with the plasma membrane is called exocytosis, also known as constitutive secretory pathway. However, specialized secretory cells have a second secretory pathway in which soluble proteins and other substances are initially stored in secretory vesicles for later release. This is the regulated secretory pathway, which is found mainly in cells that are specialized for secreting products rapidly on demand, such as hormones, neurotransmitters, or digestive enzymes (Alberts et al, 1994).
In the process of protein export, a central role is played by the signal peptide: an N-terminal segment that somehow initiates export whereupon it is cleaved from the mature protein. Three structurally dissimilar regions have been identified for signal peptides: a positively charged N-terminal region, a central hydrophobic region, and a more polar C-terminal region that seems to define the cleavage site (Heijine, 1985). Generally, signal peptides are 15-30 residues long and highly hydrophobic. Many features of the secretion pathway appear to be shared by all species, since most exported proteins can be translocated and processed similarly by the export machinery from several organisms. However, signal peptides display a remarkable lack of primary sequence homology, even among closely related proteins (Gierasch, 1989).

In eukaryotes, the first interaction of the signal peptide appears to be with the signal recognition particle (SRP). It ensures that the nascent protein chain will be correctly targeted through subsequent specific binding between SRP and its receptor in the ER membrane (Walter, 1994). This binding step occurs after the signal peptide emerges from the ribosome when a chain of about 70-80 residues has been synthesized (Heijine, 1985). It is believed that there is only one SRP in a particular organism (Gierasch, 1989). In addition to directing the protein to the ER membrane, the signal peptide also serves as a start-transfer signal, which remains bound to the translocation apparatus while the rest of the protein is threaded continuously through the membrane as a large loop. Once the C-terminal of the protein has passed through the membrane, the signal peptide is released from the translocator pore, cleaved off by the signal peptidase,
and rapidly degraded to amino acids by other proteases in the ER while the protein is released into the ER lumen (Albert et al, 1994). A signal peptide from one organism can target naturally non-secreted proteins from another organism into the protein secretion pathway (Okano et al., 1990; Liu et al., 1997).

B. Application of bioluminescent gene expression markers and the secreted *Renilla* Luciferase

Delivery of therapeutic genes to target cells and regulation of gene expression in those cells are crucial to efficient gene therapy. Regulation of gene expression is associated with an important question: how to monitor gene expression within an experimental animal and perhaps the human body? Several marker genes have been studied and well characterized for this purpose. β-galactosidase, chloramphenicol acetyltransferase (CAT), and firefly luciferase genes have been widely used in gene expression assays in both *in vitro* and *in vivo* experiments (Alam and Cook, 1990). However, functional assays of these marker genes have the same shortcoming: the removal of the transfected tissues is required to perform the assays and therefore limits the assays for gene expression to a single point in time. It would be difficult to follow gene activity once the continuity of experiment is stopped. Under certain circumstances, it would be very difficult or impossible to obtain tissue samples during clinical trials.

The use of a secreted reporter protein is a logical alternative to intracellular markers and their associated need for tissue removal. Secreted marker proteins would
permit serial evaluation of *in vivo* gene transfection in the same subject and facilitate efforts to study strategies designed to enhance the magnitude and duration of transfection efficiency.

Light emission from bioluminescence or chemiluminescence has become a powerful tool in molecular biology for detecting the presence of certain molecules as well as for studying specific cellular events. Luciferase is especially useful because the light emission assay is sensitive, simple, quick, easy to be quantified, cost effective, and generally non-toxic (Alam and Cook, 1990; Kricka, 1991). The most widely used luciferases for this purpose are from the bioluminescent *Vibrio* bacteria, from the jellyfish *Aequoria victoria*, and from the firefly *Photinus pyralis*. Recently the use of a fluorescent protein, the *A. victoria* green fluorescent protein (GFP), has permitted the direct visualization of the location of various proteins within a living cell and of the movement of living organisms within their environment (Kather *et al.*, 1995 and 1997; Presley *et al.*, 1997; Wacker *et al.*, 1997). The combination of secreted protein and bioluminescence or chemiluminescence marker is extremely attractive because it will provide a powerful tool to monitor gene transfection without destroying samples or subjects. Several secreted bioluminescence or chemiluminescence marker proteins including secreted alkaline phosphatase (SEAP) (Berger *et al.*, 1988), *Vargula hilgendorfii* luciferase (Thompson *et al.*, 1990), apoaequorin (Inouye and Tsuji, 1992), and GFP from *Aequoria victoria* (Kaether and Gerdes, 1995; Laukkanen *et al.*, 1996), have been developed. Of all these proteins only the *Vargula* luciferase is naturally
secreted, however, its substrate is too expensive (about $2,000/mg), therefore restricting its use as a reporter protein. Also restricted is the use of secreted GFP by mammalian cells, due to the very low sensitivity of the GFP assay (Kaether and Gerdes, 1995). The firefly luciferase is the most commonly used luciferase marker, but it was not functional after being fused with signal peptide from insulin for secretion (Tyedmers et al., 1996). Since it is unlikely there will be a “do it all” type reporter gene, it is necessary to develop a new marker system for different purpose under defined conditions.

In recent years, *Renilla* luciferase has emerged as a useful reporter protein and has been used as a marker of gene expression in bacteria (Lorenz et al., 1991), plants (Mayerhofer et al., 1995), yeast (Srikanta et al., 1996), and mammalian cells (Lorenz et al., 1996). The sea pansy *Renilla reniformis* (class *Anthozoa*), an anthozoan coelenterate, is a bioluminescent soft coral living in shallow coastal waters of North America. It displays blue-green bioluminescence upon mechanical stimulation (Matthews, et al., 1977). The monomeric *Renilla* luciferase [Renilla-luciferin: oxygen 2-oxidoreductase (decarboxylating), EC 1.13.12.5] catalyzes a bioluminescent reaction by oxidizing of coelenterazine in the presence of dissolved oxygen to yield oxy-luciferin, CO₂ and blue light (λₘₐₓ = 480 nm) (Matthews, et al., 1977). In *R. reniformis*, the energy of the reaction is transferred to the acceptor green fluorescent protein (GFP) which emits green light as seen in the living coral (λₘₐₓ = 509 nm). When GFP is absent, the reaction yields visible blue light (Matthews, et al., 1977). A cDNA encoding a functional full-length *Renilla* luciferase protein was previously cloned and sequenced.
Renilla luciferase is active as a nearly spherical single polypeptide chain monomer of 35 kDa with a specific activity of $1.8 \times 10^{15} \text{ hv s}^{-1} \text{mg}^{-1}$, and a turnover number of 111 μmol min$^{-1}$ μmol$^{-1}$ of enzyme (Matthews, et al., 1977). The native Renilla luciferase contains three free SH groups but no disulfide bonds. The optimal temperature for luciferase activity is 32°C, and the optimal pH is 7.4. Its activity is stimulated by the presence of salts, such as NaCl and KCl, with optimal stimulation of 2.7-fold at 0.5 M, and inhibited by many divalent cations, such as Zn$^{2+}$, Cu$^{2+}$, Fe$^{2+}$, Mn$^{2+}$. (Matthews, et al., 1977). The best substrates for Renilla luciferase are e-coelenterazine and v-coelenterazine, which are significantly more efficient than native coelenterazine, but are highly unstable at the same time (Inouye and Shimomure, 1997).

In this work, the Renilla reniformis luciferase gene product was chosen as a target protein to be engineered for secretion by mammalian cells to develop a new secreted gene expression marker for \textit{in vitro} and \textit{in vivo} studies. After being fused to the C-terminal end of the human IL-2 signal peptide, Renilla luciferase was secreted into mammalian cell culture media. Luciferase protein stability was further improved by substituting cysteine residue 152 to alanine using site-directed mutagenesis. Secreted Renilla luciferase activity was easily detected from mammalian cell culture media, but could not be detected from animal blood samples. Further modifications are necessary before secreted Renilla luciferase could be applied as an \textit{in vivo} gene expression marker.
C. Gene therapy, DNA vaccination, and gene delivery systems

Gene therapy is defined as the treatment of diseases by transferring therapeutic genes into target cells. The first gene therapy clinical trial was performed successfully in 1990 with two children suffering from adenosine deaminase (ADA) gene deficiency syndrome (Bordignon et al., 1995; Blaese et al., 1995). Since then gene therapy has become one of the most promising weapons in the battle against deadly diseases such as cancer and AIDS (Anderson, 1998), as well as autoimmune and inflammatory diseases (Seroogy et al., 1998).

Gene therapy may be a potentially revolutionary change to modern medicine. Although tremendous progress has been achieved in the fields of anesthesia, vaccination, transplantation, and radiotherapy, humans are still vulnerable to a wide variety of inherited and acquired diseases. Newly emerging diseases, such as AIDS, make it necessary to develop new forms of medical treatment. Compared to traditional therapeutic approaches, gene therapy has several advantages. For example, when compared to protein therapy, such as daily insulin injection, a gene therapy approach would eliminate the possible endotoxin contamination present during protein purification. It would also be more stable for storage and transport, and decrease the cost of preparation. Unlike radiotherapy and chemotherapy, gene therapy seeks to target specific cells or induce a specific immune response for therapeutic purposes. There are over one hundred twenty-five clinical trials that have been approved since 1990 in the United States alone, and 329 clinical studies with over 2500 patients have been initiated.
worldwide since 1989 (Gottschalk et al., 1998; Anderson, 1998). There is a wide variety of human genetic diseases, acquired diseases, and infectious diseases that have been selected for potential gene therapy treatment. The majority of gene therapy strategies have fallen into one of the following three categories: (a) genetically modified tumour vaccines, in which metastatic cancer cells from a patient are extracted, transfected ex vivo with one of several functional cytokine genes, and reinfused into the patient following irradiation to induce an enhanced target specific immune response to tumor sites throughout the body, (b) direct local gene delivery, which is defined as direct injection of gene vectors into target cells, (c) systemic directed gene therapy with subsequently directed transfection of target tissues (Taneja et al., 1995). So far, however, no single gene therapy approach has definitively improved the health of a single one of the more than 2,500 patients who have enrolled in gene therapy trials worldwide (Anderson, 1998). The majority of gene therapy trials are still in Phase I (evaluation of safety) or II (preliminary evaluation of efficacy), and only one reached Phase III (evaluation of efficacy). Although there are some promising results, the inconsistency of clinical trials, limited knowledge of in vivo gene expression and host immune response, and lack of ideal gene delivering system are among the main reasons for the relative failure of gene therapy so far.

During recent years, another gene therapy strategy, DNA vaccination, also known as genetic or polynucleotide immunization, has become a major focus of attention. DNA vaccination is defined as the delivery of plasmid DNA encoding either individual or a
collection of antigens specific to a particular pathogen to a host. The DNA is then taken in by host cells and the gene(s) is expressed. The foreign protein is produced within the host cells and then processed and presented appropriately to the immune system, inducing a specific immune response (Cohen et al., 1998). The major difference between DNA vaccination and traditional gene therapy approaches is that the goal of DNA vaccination is to provoke a host immune response, not to alter or replace the function of certain genes. Therefore, it may not be necessary to reach high gene expression/protein production levels in DNA vaccination, because even small amount of antigen can theoretically stimulate a protective immune response. Although traditional vaccination strategies, such as live, attenuated or whole, inactivated agents, purified or recombinant subunit proteins and synthetic peptides have been very successful in the past, they are often hampered by toxicity, the presence of infectious agents, weak immune responses, and prohibitive costs, especially in the developing world (Gregoriadis, 1998). Another major disadvantage of traditional vaccines is time. The preparation of viral vectors, production and purification of recombinant proteins from bacteria, yeast or stably transfected mammalian cell lines can easily take months to develop, and even years in some cases (Davis et al., 1995). In addition, DNA vaccination offers an extremely powerful tool for molecular immunologists to study the immune system and with which to develop new vaccines and other immunotherapeutic approaches. One can easily and rapidly clone and modify genes in plasmid DNA expression vectors, allowing many new constructs to be produced and tested in a short period of time which can be on the order of weeks. In
animal model systems, DNA vaccines have proved to be as effective as traditional vaccines, and in some cases, even better (Wahren et al., 1997; Davis et al., 1995).

Since DNA vaccines are taken by host cells, and antigens are synthesized inside of cells, it is not surprising to find that DNA vaccination provokes strong and persistent cell-mediated and humoral immune responses to the antigen encoded by the plasmid (Davis et al., 1995). The endogenous synthesis of proteins leads to the induction of a cytotoxic T lymphocyte (CTL) response via the major histocompatibility complex (MHC) class-I-restricted pathway. Meanwhile, synthesised proteins are released extracellularly through cell lysis. It is believed that the extracellular releasing of antigen primes the induction of a humoral response, as well as a helper T lymphocyte (Th2) response via MHC class-II- restricted antigen presentation by antigen-presenting cells (APCs) that have taken up the foreign antigen (Cohen et al., 1998).

A variety of routes of administration and methods of DNA vaccination including the use of the “gene gun”, aerosol delivery, and intramuscular DNA injection have been developed (Lai et al., 1998). It is unlikely that there will ever be an universal method, but rather there would be multiple approaches taken specifically for certain organ sites and diseases (Wivel et al., 1998). Skeletal muscle has been the preferred target cell for DNA vaccination because of its large mass, vascularity, and accessibility (Davis et al., 1995). Since muscle fibers are nondividing, effective gene delivery could potentially result in long-lived protein production (Kessler et al., 1996). Other organs, such as liver (Malone et al, 1994), central nervous system (CNS), lung, kidney, and tongue (Prigozy
et al., 1993), have also been selected as targets for DNA injection. Gene expression was also reported in progeny after injection of plasmid DNA (Tsukamoto et al., 1995). DNA vaccination has been applied to induce immune response against Influenza A (Montgomery et al., 1993; Yankauckas et al., 1993), Hepatitis C (Geissler et al, 1997), Hepatitis B (Mancini et al., 1995; Michel et al., 1995; Davis et al., 1995; Gramzinski et al., 1998), HIV (Wang et al., 1993), malaria (Mor et al., 1995), rabies (Xiang et al., 1995), and most recently, Ebola (Xu et al., 1998). There are reports of DNA vaccination being used for the treatment against tumor (Codon et al., 1996) and autoimmune diseases (Youssef et al., 1998). Recent reports showed that delivering plasmid DNA encoding cytokine (Nitta et al., 1998) or autoimmune antigen (Liu et al., 1998) through intramuscular (IM) injection could prevent type I diabetes or reduce insulitis in non-obese-diabetic mice. Studies have shown that DNA injection is not likely to initiate or accelerate the development of systemic autoimmunity (Wolff et al., 1993). DNA vaccination protocols have recently entered the clinic for initial safety and immunogenicity testing in humans against colon cancer (carcinoembryonic antigen) and cutaneous T cell lymphoma (T cell receptor Vβ) (Horn et al., 1995; Cohen et al., 1998).

A major technical obstacle for gene therapy/genetic vaccination is to find an ideal delivery system. Many different gene-delivering systems have been applied in gene therapy, and they can be divided into two groups: viral and nonviral vectors. Each type of vector system has its own advantages and disadvantages. Among viral vectors, retroviral vectors were the first ones to be applied in gene therapy trial and are the most
extensively studied vectors (Mavilio et al., 1994; Miller et al., 1992). Retroviral vectors can integrate into host genomes and offer possible long-term gene expression. However, retroviral vectors are unable to infect postmitotic cells, such as mature myofiber, and are limited to carrying relatively small segments of DNA (no bigger than 7 kb). In addition, retroviral vectors present general disadvantages, such as the possibilities of carrying over of adventitious microorganisms, insertional mutagenesis and/or disruption of host genome, and replication-competent (helper) retroviral production. Adenoviral vector is another commonly used viral vector, and currently is the primary candidate for direct in vivo gene delivery, but also is limited by size of DNA is can carry. Compared to the retroviral vector, adenoviral vectors are considered safer, have a larger capacity for foreign genes (up to 40 kb), and will not integrate into host genome. Adenovirus is also able to infect nonreplicating cells, including skeletal muscle in vivo (Quantin et al., 1992). The drawbacks are that genes may have transient function due to lack of integration, and there is a strong host immune response against the viral proteins (Miller et al., 1992). Several other viral vectors are being explored: adeno-associated viruses, herpesviruses, alphavirus, lentiviruses, and poxviruses. None of these viral vectors is perfect, and each is likely to have its own therapeutic advantages.

The known and unknown side effects of viral systems have encouraged the development of nonviral delivery system (Gao et al., 1995). Compared to viral vectors, nonviral vectors usually do not integrate into the host genome, and gene transfer efficiency is, in general, lower when compared with viral vectors. Liposomes, which are
small spheres composed of lipid bilayers, were the first nonviral delivering vehicle, but are considered too inefficient for gene therapy (Felgner, 1997). The DNA-lipid complex eliminates the introduction of virus particles but demands large amount of DNA and transfection efficiency is lower compared to virus vectors. Another shortcoming of liposomes is that because of mismatch between liposome and plasmid (0.025–0.1 micron versus 2 microns), only a small portion of plasmid (less than 1%) can be encapsulated when liposomes are synthesized in the presence of plasmids. Cationic lipids have been applied to replace simple lipids for packaging of plasmid DNA. These new lipids, named lipoplexes, are more variable and complicated than simple liposomes and lead to higher gene expression level (Felgner, 1997). Combined with the use of gene gun, the gene delivering efficiency of lipoplexes can be greatly increased (Felgner, 1997). However, the liposomes/lipoplexes systems are generally considered much less efficient compared to viral vector (Crystal, 1995). As the initial route of administration for DNA vaccination, intramuscular (IM) injection of naked DNA started to be developed as a simple and safe way to deliver foreign genes around the late 1980’s. IM injection was first reported for transferring genes into skeletal muscle in vivo by direct injection of plasmid DNA without any delivery vehicle, such as a lipid shell (Wolff et al., 1990). Intramuscular DNA delivery has been used to express reporter genes in animal models. Several reporter genes, such as Firefly luciferase (Wolff et al., 1990; Levy et al., 1996; Perez et al., 1996; Prigozy et al., 1993), chloramphenicol acetyltransferase (Wolff et al., 1990; Wells, 1993; Prigozy et al., 1993), β-galactosidase (Wolff et al., 1990; Nishi et al.,
1996; Prigozy et al., 1993), and human placental alkaline phosphatase (Perez et al., 1996) have been expressed in adult and neonatal rat (Wolf et al., 1991), primate skeletal muscle (Jiao, et al., 1992), cardiac muscle (Lin et al., 1990) and even in progeny (Tsukampoto et al., 1995).

Compared with viral vectors, “naked” gene transfer offers several advantages (Levy et al., 1996; Davis et al., 1993). “Naked” DNA gene transfer strategies do not require dividing cells, has virtually no size limitation, and should be less likely to create immunological problems, since plasmid DNA can be purified to homogeneity. Despite evidence that most of the DNA remains in an unintegrated circular form, which minimizes the risk of genomic insertional mutagenesis, expression has been shown to persist for up to 19 months (Wolff et al., 1992). Studies have shown that in mouse skeletal muscle, plasmid DNA transfer is more efficient than that of adenovirus and retrovirus (Davis et al., 1993).

There are several factors affecting the efficiency of “naked” DNA transfer and the stability of its expression should be considered during DNA injection. Degeneration of muscle, preinjection of sucrose (Davis et al., 1993; Wells and Goldspink, 1992), and preinjection of dexamethazone (Malone et al., 1994) have been found to be able to improve the distribution of injected DNA and gene expression, although the mechanism behind the effects remain unclear. It is believed that DNA expression in muscle is dose-dependent, volume-dependent (larger volume gives better expression and less variation), and that muscle cells can be saturated by DNA injection (Davis et al, 1993). Direct gene
transfer into mouse muscle in vivo was also shown to be affected by age and gender. It is believed that 5-6 weeks old male mice are optimal for “naked” DNA injection (Wells and Goldspink, 1992).

D. Application of engineered secreted proteins in gene therapy and DNA vaccination

Most current gene therapy protocols require genetic modification of autologous cell (Hughes et al., 1994). Unfortunately, not all kinds of autologous cells are suitable to be genetically modified and most of these modification procedures are labor intensive, time consuming and costly (Hughes et al., 1994). Although injection of therapeutic protein has been successful in several cases, for example, daily injection of insulin in treatment of diabetes type I, it is not a viable option to cure disease, considering the cost of preparation, and the side-effects such as hypoglycemia and dawn phenomenon (Fenjves et al., 1994). Another approach, cell therapy, has been hampered by the difficulty to maintain therapeutic cells in the recipient due to host immune rejection. Even without host rejection, the availability of donors for therapeutic cells remains as a significant concern for cell therapy (Gottschalk et al., 1998).

Alternatively, genes may be delivered to tissues which serve as sites for the synthesis and secretion of proteins that have effects elsewhere in the body. Secreted protein should be exposed throughout the body following the bloodstream, raising the possibility to genetically modify certain autologous cells, release therapeutic protein, and reach certain cells or organ, which otherwise would be difficult to be modified. For
instance, this would be helpful for cancer patient at metastatic stage. If an organ or
tissue-specific-targeting-signal can be added to therapeutic secreted protein, it will make
gene therapy of secreted protein even more attractive. Although the duration of gene
expression and protein circulation may vary in different cases, it was reported that
secreted human erythropoietin (Epo) protein could be detected in circulation for at least
10 weeks after single intramuscular DNA injection (Kessler et al., 1996). The long-term
secretion of β-glucuronidase, a therapeutic protein for the treatment of human
mucopolysaccharifosis (MPS) type VII, was also achieved in mice skeletal muscles cells
(Naffakh et al., 1996).

Many naturally secreted proteins have been applied in gene therapy and gene
vaccination to treat a wide variety of diseases. For example, human erythropoietin (Epo)
(Kessler et al., 1996; Tripathy et al., 1994), human α1-antitrypsin (Mastrangeli et al.,
1994), human apolipoprotein E (Fenjves et al., 1994), human IL-10 (Nitta et al., 1998),
human IL-12 (Tahara and Lotze, 1995), human growth hormone (hGH) (Anwer et al.,
1998), hepatitis B surface antigen (Davis et al., 1993), hookworm larval antigen
anlylostoma secreted protein (ASP) (Hotez et al., 1996), and human vascular endothelial
growth factor (VEGF) 165 (Muhlhauser et al., 1995) have been secreted into the
circulation of experimental animals and induced corresponding biological responses.

However, there are many proteins with therapeutic potential that are not naturally
secreted. For example, the retinoblastoma (RB) protein, a tumor suppressor implicated
as a regulator of several cellular processes, was injected into the blood stream causing
tumor regression in humans (Welch and Wang, 1995). The autoimmune antigen glutamic acid decarboxylase (GAD) 65 is another example. It was reported that intrathymic and intravenous injection of the purified GAD65 protein into non-obese mice (NOD) could protect NOD mice from the development of insulin-dependent diabetes mellitus (IDDM) (Kaufman et al, 1993; Tisch et al, 1993). Both retinoblastoma and GAD65 are not naturally secreted, and it may open new directions in cancer therapy and autoimmune research if retinoblastoma and GAD65 can be engineered to become secreted proteins, and mimic the result from that of protein injection. Engineering the in vivo secretion of protein may provide more stable and longer-lasting therapeutic protein levels than that from the injection of purified proteins, and should be economically more advantageous and less painful physically and psychologically to the patients compared to protein injection.

In general, a leader sequence encoding a hydrophobic signal peptide is used to generate secreted protein. The signal peptide will redirect naturally non-secreted protein to the protein secretion pathway, eventually causing it to be secreted by the host cell. Several therapeutic proteins, including human adenosine deaminase (ADA) (Hughes et al., 1994), acidic fibroblast growth factor (aFGF) (Tabata et al., 1997), human thyrotropin receptor (Chazenbalk et al., 1997), and human leukocyte elastase (HLE) (Okano et al., 1990), have been engineered to become secreted proteins for therapeutic purposes. It was reported that artificially secreted proteins are immunologically and often catalytically similar to their intracellular forms (Hughe et al., 1994).
E. Insulin-dependent type I diabetes: Glutamic Acid Decarboxylase (GAD65) as an autoantigen, and the NOD mouse as an animal model

Diabetes is the seventh leading cause of death by disease in the United States (Stryer, 1988). There are two types of diabetes: Type 1 is an autoimmune disease where the body does not produce any insulin; Type 2 is a metabolic disorder resulting from the body’s inability to make enough, or properly use, insulin. It has been reported that the incidence of diabetes mellitus in industrialized countries is about 1%, and there are 15.7 million people or 5.9% of the population in the United States who have diabetes (Stryer, 1988). While an estimated 10.3 million have been diagnosed, unfortunately, 5.4 million people are not aware that they have the disease, and approximately 2,200 people are diagnosed with diabetes every day. Based on death certificate data, diabetes contributed to more than 187,000 deaths in 1995. A total of 75,000 workers were reported to be permanently disabled because of diabetes in 1997. The total annual economic cost of diabetes in 1997 was estimated to be $98 billion dollars, including $44.1 billion in direct medical and treatment costs, and $54 billion for indirect costs attributed to disability and mortality. Although diabetes studies and medical research have made tremendous progress during the last decade, diabetes still remains a chronic disease with no cure. Diabetic patients require daily insulin injections to stay alive.

The mechanism behind diabetes is not clear. Diabetes Type I is considered as an organ-specific autoimmune disease, resulting from a failure to develop or maintain immune tolerance to self-antigens expressed in the islets of Langerhans (Winter et al,
1997; Andrea et al, 1992). Although both T cells and B cells are involved, the destruction of pancreatic β cells is mediated predominantly by the activity of CD4+ and CD8+ T cells (Lernmark et al, 1994). During the normal development of T cells in the thymus, self-antigen-reactive T cells undergo clonal deletion or clonal anergy, which effectively removes these cells from the pool of mature antigen reactive T cells (Alberts et al, 1994). In general, T-cell reactivity against self-antigen expressed on β cells does not occur because of the failure to activate T cells reactive with the antigen, termed clonal ignorance. This may be broken with viral infection or cytokines. In one transgenic model, dendritic cells surrounding the islets of Langerhans have been shown to be responsible for presentation of islet antigens to the immune system. B-cell tolerance can also involve mechanisms of clonal deletion or clonal anergy similar to that of T cells.

In the case of Diabetes Mellitus Type I, the failure of immune tolerance leads to total destruction of pancreatic β cells, and insulin production is subsequently stopped. The development of diabetes can be divided into three stages. First, immune ignorance against autoimmune antigens (GAD65, insulin etc.) presented by pancreatic β-cells is suppressed, and self-reactive T and B lymphocytes recognize these antigens. Second, the activation of the Th1 cell population leads to the infiltration of pancreatic β-cells by macrophage, CTL, and autoantibody-producing B-cells resulting in insulitis. Third, insulin production is stopped due to extensive pancreatic tissue damage. It is believed that both cellular and humoral type of host immunity are involved in the development of diabetes, and balance shifting between Th1/Th2 cell population plays an important role in
diabetes (Parish et al., 1995). Insulitis is a crucial stage during the development of diabetes, and protection of pancreatic tissue from insulitis is a good indication for the protection of diabetes (Herold et al., 1992; Andrea et al., 1996).

The clinical syndrome of human diabetic patients starts with increased urinary glucose level (glucosuria > 10.0 mM throughout the day and night). As the disease progresses, hyperglycemia, visual blurring, and weight loss are the first symptoms found in diabetic patients. The symptoms of diabetes complication include: diabetic retinopathy (retinal microangiopathy), diabetic nephropathy (microangiopathy involving the kidney, a major cause of morbidity and mortality), diabetic neuropathy (disordered function of somatic or autonomic nerves), coronary artery disease, peripheral vascular disease (diabetic foot, skin damage, etc), and diabetic ketoacidosis (Felig et al., 1987). Several clinical treatments have been established for diabetes with daily injection of insulin as the main treatment. Insulin is injected twice a day or administered with a portable pump. However, there are several complications with insulin treatment that can cause severe side effects in patients: hypoglycemia, Somogyi phenomenon (rebound or reactive hyperglycemia and ketinuria after excessive insulin administration), dawn phenomenon (early-morning rise in blood glucose concentration), insulin lipodystrophy (poor insulin absorption of subcutaneous tissues), insulin allergy, resistance to insulin, and insulin-induced edema. The quality of human insulin products also presents a problem for insulin treatment. Dietary management is another form of treatment for diabetic patients, but successful implementation of diet is only achieved occasionally (Felig et al., 1987).
Besides daily insulin injection, transplantation of islet cells or pancreas tissue into diabetic patients has also shown some encouraging results since 1986. However, extremely high cost (up to $100,000/patient) and strong host immune rejection, have to be overcome before cell/tissue transplantation can be widely applied to diabetic patients.

Gene therapy would be an attractive approach to prevent or treat diabetes if it could restore host immune tolerance through genetic vaccination or provide physiological levels of insulin. Up to now, most studies were conducted in NOD mice, an animal model for IDDM. Several genes have been selected for genetic vaccination of diabetes: insulin (Kolodka et al., 1995), glutamic acid decarboxylase (GAD) 65 (Kaufman et al., 1993; Tisch et al., 1993), and GAD67 (Elliott et al., 1993). Among these autoantigens, GAD65 protein is one of the earliest recognized by autoimmune antibodies in IDDM (Peterson et al., 1994). The recognition of GAD65 is believed to be the trigger of other autoimmune reactions (Kaufman D. L. et al., 1993. Tisch R. et al., 1993; Aizpurua et al., 1994). Therefore if immune tolerance against GAD65 can be restored, there is a possibility of delaying the development of diabetes.

GAD is the biosynthetic enzyme for \( \gamma \)-aminobutyric acid (GABA), an inhibitory neurotransmitter in the central nervous system (Erlander and Tobin, 1991). GABA is also found outside of the central nervous system, in the liver, oviduct, adrenal, sympathetic ganglia, gastrointestinal tract, circulating erythrocytes, testis, ovary, kidney and pancreatic islets (Tillakaratne-NJ et al., 1995). GAD exists in two isoforms: GAD67 and GAD65, encoded by two different genes on different alleles. GAD67 is a 67
kilodalton (KDa) protein found in the cytosol (Erlander and Tobin, 1991). The *gad67* gene is located on human chromosome 2, contains 16 exons and covers a 45 kb region. GAD65 is an amphiphilic and membrane-anchored protein with a molecular weight of 65 kDa. The *gad65* gene is located on human chromosome 10 with 16 exons and covers a 79 kb region (Bu and Tobin, 1994). There is 65% amino acid sequence homology between GAD67 and GAD65, with low-homology at the N-terminus (Li, *et al.*, 1995).

The cDNA encoding the GAD65 protein has been cloned, and both DNA and protein have been thoroughly studied. The majority of epitopes associated with IDDM have been located at the C-terminal and the middle region of the protein (Richter *et al.*, 1993; Ujihara *et al.*, 1993). No epitope is found at the N-terminal region (Richter *et al.*, 1993). After being synthesized, the GAD65 protein goes to the endoplasmic reticulum and Golgi apparatus, and undergoes a posttranslational modification step called palmitoylation (Christgau *et al.*, 1992). The palmitoylation modification adds a 16-carbon saturated fatty acid to the N-terminus of GAD65 protein. GAD65 then becomes an N-terminal membrane-anchored protein found on the surface of microvesicles (Christgau *et al.*, 1992). It has also been shown that amino acid residues 24-31 and a signal located within amino acid 1-27 of GAD65 are crucial for GAD65 membrane anchoring (Shi *et al.*, 1994; Solimena *et al.*, 1994).

In addition to IDDM, another human disease, stiff-man syndrome (SMS), is also associated with autoimmunity to GAD (Erlander and Tobin, 1991; Solimena *et al.*, 1991). The GAD65 antigen is the most often targeted isoform for IDDM and SMS, even though
different GAD65 epitopes are recognized in the two diseases (Hagopian et al., 1993). Injections of GAD65 purified protein in NOD mice, which is the established animal model to study human diabetes, have been shown to delay the development of insulitis and diabetes (Kaufman et al., 1993; Tisch et al., 1993). Because of the high cost with protein isolation, and of the impossibility to thoroughly eliminate endotoxin contamination during protein purification, the vaccination against injection of GAD65 protein does not appear to be the most practical solution for the treatment of diabetes.

The non-obese diabetic (NOD) mouse is an important animal model for human IDDM. The NOD mouse was first established as an inbred strain in 1974 at the Shionogi Research Laboratories in Osaka, Japan (Hanafusa et al., 1994). It was derived from a subline of the CTS strain on a JCL-ICR background, during selective breeding searching for a new animal model of diabetes. Interestingly, the NOD was derived from a normal glycemic subline instead of a hyperglycemic one (Hanafusa et al., 1994).

The NOD mouse is an excellent animal model for human IDDM because it can spontaneously develop IDDM with a similar immunopathological profile to the human disease (Jaramillo et al., 1994; Shieh et al., 1993; Hanafusa et al., 1994). It has been shown that the development of IDDM in NOD mice is under polygenic control and is influenced by many environmental factors (Bowman et al., 1994). Generally 60-80% of females and 20% of males show accumulative diabetes incidence by 30 weeks of age (Hanafusa et al., 1994. Aizpurua et al., 1994. Baxter et al., 1991). Although promising data were obtained through experiments conducted on NOD mice (French et al., 1998;
Kolodka et al., 1995), we are still far away from controlling the disease in either mice or humans.

Many key features of human insulin-dependent diabetes (IDD) are observed in the NOD mouse: the development of insulitis, whereby pancreatic islets of Langerhans are infiltrated by lymphocytes that are selectively cytotoxic to the insulin-producing β cells, the inheritance of particular major histocompatibility complex (MHC) class II alleles, representing the major component of genetic susceptibility, the transmission of IDDM by hematopoietic cells in bone marrow, and the T cells dependence of disease pathogenesis (Bowman et al., 1994). There are also some minor differences between human and mouse disease. The presence of a high percentage of T cells in NOD lymphoid tissues and peripheral blood distinguishes NOD mice from human with IDDM. NOD mice do not display the severe diabetic ketoacidosis character as do untreated human patients with IDDM, perhaps due to an enhanced ability of mice to metabolize blood ketones to lactate. In addition, NOD mice exhibit a pronounced female gender bias for disease susceptibility that is not seen in humans (female: male ratio is about 4:1 in NOD mice and 1.7:1 in human patients). While the specific reasons for this difference are unclear, female mice are known to show stronger immunological responses to exogenous stimuli than males and it has been demonstrated that the lower incidence of IDDM in NOD males is partially regulated by gonadal sex steroids. Finally, the pathogenesis of IDDM in NOD mice is associated with expression of endogenous defective retrovirus in β cells, a feature not seen in human (Bergman et al, 1994).
A major difference must be considered when comparing IDDM in human and NOD mice (Bowman et al, 1994). Given the genetic heterogeneity within the human population, the development of IDDM in NOD mice is likely to reflect heterogenous mixtures of susceptibility genes whose penetrance are responsive to different NOD mice that inherit the same gender-specific set of susceptibility genes, a consequences of over 50 generations of sibling matings. The penetrance of these genes can be analyzed under constant, well-defined environment conditions of diet, temperature and exposure to pathogenic agents. Consequently, the natural history of IDDM development in a well-maintained NOD colony is quite predictable. Indeed, an intervention study in NOD mice can be designed in such way that the treatment is initiated at a presymptomatic stage prior to the occurrence of insulitis (up to 3 weeks postpartum), or before the onset of diabetes (4 to 8 weeks postpartum), or before the onset of disease, at a time when considerable numbers of β Langerhans cells are still intact. By contrast, the genetic and environmental heterogeneity associated with the natural history of IDDM in humans is such that the age of disease onset is extremely broad and may occur beyond 50 years of age. Given these complexities, it has been difficult for clinical investigators to develop simple diagnostic tools for the early identification of humans destined to develop IDDM. For these reasons, studies to prevent IDDM in NOD mice must be carefully analyzed for their applicability to the therapeutic intervention in human disease (Bowman et al, 1994).

The NOD mice used in this project, NOD/MrkTacBR, were purchased from the Taconic Lab. This strain was derived by Taconic in1985, and an inbred foundation
colony has since been maintained in genotobiotic isolators. The NOD mouse was developed from non-inbred ICR mice and subsequently inbred by Makino for over 20 generations. Diabetes development in NOD/MrkTacBR mice is characterized by insulitis, defined as leukocytic infiltration of the pancreatic islets. Marked decreases in pancreatic insulin content occur in females at about 12 weeks of age (Gaskins, *et al.*, 1992) and several weeks later in males. Onset of diabetes is marked by moderate glycosuria (1+ reading on Lily Tes-Tape™) and the non-fasting plasma glucose higher than 250 mg/dl. It is best to begin monitoring for development of glycosuria at weekly intervals starting at 10 weeks of age. The incidence of diabetes in NOD/MrkTacBR females and males are currently at 85% and 50% respectively at 39 weeks of age.

In this work, a secreted form of GAD was engineered to evaluate its biological effect on diabetes in NOD mice. Intramuscular DNA injection was conducted to inhibit the development of diabetes in NOD mice. Results showed that IM injection of both intracellular and secreted forms of GAD dramatically reduced the development of insulitis compared to control groups.
CHAPTER TWO

MATERIALS AND METHODS

A. Reagents

Double-distilled water (Nanopure II System, Barnsted/Thermolyne Corp., Dubuque, Iowa) and commercially available chemicals were used to make all solutions. Solutions and glassware were autoclaved (AMSCO Eagle Series, Steris Corporation, Eire, PA, USA) if necessary. All reagents for mammalian cell culture are purchased from Sigma Chemical Co. (St. Louis, MO), ICN Biomedical Inc. (Aurora, Ohio), and Life Technologies Inc. (Grand Island, NY). Culture media were kept at +4 °C, and −20 °C for long time storage.

B. Gene expression vectors used in this project

Two retroviral vectors, pLNCX and pLXSN, were kindly presented by Dr. Miller from the University of Washington. The retrovirus vector pLNCX (Figure 1A) is a double stranded (ds) DNA cloning vector that can transfer and express foreign genes in dividing mammalian cells (Miller et al., 1989). The sources of pLNCX vector include moloney murine leukemia virus DNA, moloney murine sarcoma virus DNA, human cytomegalovirus DNA, and transposon Tn5 DNA. The full length of pLNCX vector is 6620 bp. A neomycin gene is transcriptionally controlled by the 5' moloney murine sarcoma virus (Mo-Musv) long terminal repeat (LTR) promoter and will be used as a
positive selectable marker for transfection of mammalian cells. A multiple cloning site is available for cloning of foreign DNA and the cloned foreign DNAs will be under the transcriptional control of cytomegalovirus (CMV) promoter. There is a poly A signal in the 3' LTR (Miller et al., 1989).

Retrovirus vector pLXSN (Figure 1B) is a 5874 bp double strand DNA cloning vector for gene transfer and gene expression (Miller et al., 1989). Its structure is very similar to that of vector pLNCX except that the cloned foreign DNA in pLXSN vector is transcriptionally controlled by the 5' LTR instead of a CMV promoter.

Another mammalian cell expression vector, pND2 (Figure 1C), was chosen as a gene delivery vector for animal studies. pND2 was developed by Dr. Rhodes at University of California at Davis and Dr. Malone at University of Maryland at Baltimore. The foreign genes cloned into pND2 vector are under transcriptional control of CMV promoter. A CMV promoter enhancer and an intron in pND2 can increase the gene expression level. An Ampicillin gene is used as selection marker for DNA transformation in E.coli. pND2 vector has been applied for in vitro and in vivo studies previously and high levels of gene expression have been reached (Dr. Malone, personal communication).

Preliminary data showed that the CMV promoter is much stronger than LTR promoter in LMTK(0) and COS cells. Compared to pLNCX vector, the result of in vitro studies showed that PND2 vector has 15 to 50 times higher gene expression level than that from pLNCX (Liu et al, unpublished data). Therefore the pLNCX vector was selected as
gene transfer vector for *in vitro* study, and pND2 was used for *in vivo* study. All published results and data are based on the studies conducted on pLNCX retroviral vector and pND2 vector.
Figure 1. Partial structures of gene transfer vectors used in this study. Ampicillin resistance gene and bacterial origin of replication are not shown. LTR, long terminal repeat; \textit{neo}, neomycin resistance gene; pA, polyadenylation site; MCS, multiple cloning site (5’ to 3’: \textit{SalI} – \textit{BglII} – \textit{NarI} – \textit{NheI} – \textit{ClaI} – \textit{SmaI} – \textit{BamHI}); SV, simian virus 40 promoter; HCMV IE1, the CMV immediate early promoter.
A. pLNCX

```
LTR  ---  NEO  ---  CMV  ---  LTR
      |                  |
      v                  v
     pA                  pA

LTR

HindIII  HpaI  Clal

B. pLXSN

```

```
LTR  ---  SV  ---  NEO  ---  LTR
      |                  |
      v                  v
     pA                  pA

LTR

EcoRI  HpaI  XhoI  BamHI

C. pND2

```

```
HCMV IE1  ---  HCMV intron  ---  MCS
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pA
C. DNA sequence analysis and oligonucleotide synthesis

DNA sequencing and oligonucleotides synthesis were performed using a 373A DNA Sequencer (Applied Biosystems, Inc.) and a 394 DNA/RNA Synthesizer (Applied Biosystems, Inc.) respectively, at the Center for Molecular Biology and Gene Therapy, Loma Linda University.

The PRISM Ready Reaction DeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.) was used for DNA sequencing. DNA templates (OD_{260/280} > 1.8) were prepared with double-distilled water at a concentration of 200 μg/μl.

Reaction solution was prepared by mixing 9.5 μl terminator mix, 5.0 μl DNA template, and 3.2 pmol DNA primer in 600 μl centrifuge tube. The final volume was adjusted to 20 μl with water. The reaction was set up as follows: preheating to 96°C, 96°C for 10 seconds, 50°C for 5 s, 60°C for 4 minutes, and total for 25 cycles. Samples were purified with Centri-Sep columns as follows: columns were hydrated with 800 μl of double-distilled water for 30 minutes, centrifuged at 750 x g for 2 minutes, and samples were then loaded to the center of the column. Samples were collected from columns after being centrifuged at 750 x g for 2 minutes, air dried, and resuspended with 4 μl of loading buffer (5 μl deionized formamide, 1 μl 50 mM EDTA, pH 8.0). Samples were placed on ice after being denatured at 90°C for 2 minutes, and were loaded on a 373A DNA Sequencer.
D. Restriction enzyme analysis of DNA

Restriction endonucleases from Boehringer Mannheim Corporation (Indianapolis, IN) were used to digest plasmid or genomic DNA, according to the manufacturer’s instruction. Generally, 500 ng of DNA were digested by 1 unit of enzyme for 2 hours at final volume of 30 μl. DNA samples were analyzed by 0.8% agarose gel electrophoresis.

E. DNA ligation

T4 DNA ligase (Boehringer Mannheim Corporation, Indianapolis, IN) was used for ligation of DNA fragments, according to the manufacturer’s instruction. For DNA ligation between cohesive ends, 500 ng of total DNA was used in a final volume at 20 μl, with a vector-to-insert ratio of 1:3. For DNA ligation between blunt ends, 1 ng of total DNA was used in a final volume at 20 μl with a vector-to-insert ratio of 1:5. Ligation reactions were incubated at 17 °C overnight, and 10 μl aliquots of ligation mixture were used to transform *E. coli* competent cells.

F. Gel electrophoresis of DNA

To analyze DNA samples after restriction enzyme digestion, 1% agarose (wt/vol) dissolved in TAE buffer (50mM Tris base, 50 mM boric acid, 1mM disodium EDTA, pH 8.0) was used. The samples were mixed with 1/10 volume of 5x loading buffer (30% glycerol, 1.8x TBE, pH 8.0, 0.06% bromophenol blue, 60 mM disodium EDTA, 0.12% mg/ml DNAase-free Rnase), loaded in wells, and were subjected to a 90 V current
for 45 minutes. Gels were stained with ethidium bromide (0.5% µg/ml, final) for 5 minutes before being visualized under ultraviolet (UV) light.

To detect small DNA fragments (< 250 bp), SDS-polyacrylamide gel was used. To make 5 ml 12% resolving gel, 1.6 ml of H₂O, 2 ml of 30% acrylamide mix (29 g acrylamide and 1 g N, N'-methylenebisacrylamide in 100 ml H₂O), 1.3 ml of 1.5 M Tris (pH 8.8), 50 µl of 10% SDS, 50 µl of 10% ammonium persulfate, and 2 µl of TEMED were mixed. To make 2 ml 5% stacking gel, 1.4 ml of H₂O, 330 µl of 30% acrylamide mix, 250 µl of 1.0 M Tris (pH 6.8), 20 µl of 10% SDS, 20 µl of 10% ammonium persulfate, and 2 µl of TEMED were mixed. The samples were mixed with 1/10 volume of 5x loading buffer, loaded in wells, and were subjected to a 125 V current for 60 minutes. Gels were stained with ethidium bromide (0.5% µg/ml, final) for 5 minutes before being visualized under ultraviolet (UV) light.

G. Mini-prep of plasmid DNA by boiling method

Bacterial cells were seeded into 2 ml LB medium with 100 µg/ml ampicillin and grown at a 37°C shaker overnight. The 2 ml cultures were then transferred into 2 ml Eppendorf tubes and spun down at 10,000 x g for 5 minutes. The pelleted cells were suspended in 200 µl of double distilled water (ddH₂O) and 200 µl of 2 x lysis solution (16% sucrose, 1% Triton X-100, 100 mM EDTA, and 20 mM Tris-HCl, pH 8.0). Twenty-five microliters of lysozyme solution (10 mg/ml in H₂O) were added to each tube. Tubes were vortexed well and boiled in a water bath for 40 seconds, then spun
down at 12,000 x g for 10 minutes at room temperature. The debris at the bottom of each tube was removed with a toothpick. DNA was precipitated with 40 µl 3M NaAC and 400 µl of isopropanol. DNA was resuspended in H$_2$O and loaded on gel directly to determine the MW. Samples were then taken for restriction enzyme analysis.

**H. Maxi-prep of plasmid DNA using alkali lysis method**

Thirty milliliters of bacteria culture carrying a plasmid of interest were grown to late log phase, and 25 ml of culture were inoculated into 500 ml of LB medium containing the appropriate antibiotic in a 2-liter flask. The culture was grown for about 12-16 hours at 37°C with vigorous shaking (300 cycles/minutes on a rotary shaker). Bacterial cells were harvested from 500 ml culture by centrifugation at 6000 rpm for 15 minutes at 4°C in a Sorvall GS3 rotor (Beckman, Palo Alto, CA). The supernatant was discarded, and the bottle was kept in an inverted position to allow all of the supernatant to drain.

The cell pellet was resuspended in 10 ml of Solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0). One milliliter of freshly prepared solution of lysozyme (10 mg/ml in 10mM Tris-HCl, pH 8.0) and 20 ml of freshly prepared Solution II were added into cell suspension, thoroughly mixed, and stored at room temperature for 10 minutes. Fifteen milliliters ice-cold Solution III (60 ml of sodium acetate, 11.5 ml acetic acid and 28.5 ml H$_2$O mixed together) were added and mixed well, stored on ice for 10 minutes.
Bacterial lysates were spun down at 8000 rpm for 15 minutes at 4°C in a Sorvall GS3 rotor. The supernatant was filtered through four layers of cheesecloth in a 250 ml centrifuge bottle. Isopropanol was added to solution of 0.6 volume ratio, mixed well and stored at room temperature for 10 minutes. The nucleic acids were recovered by centrifugation at 8000 rpm for 15 minutes at 4°C in a Sorvall GS3 rotor. The supernatant was removed carefully and the open bottles were inverted to allow the last drops of supernatant to drain. Five ml 70% ethanol were used to wash the pellet twice before the pellet was dried in open air for 5 minutes. DNA pellet was then dissolved in 2 ml TE (pH8.0) and stored at -20°C.

I. Purification of closed circular DNA by equilibrium centrifugation in CsCl-Ethidium gradients

*E.coli* cells transformed with DNA constructs of interest were grown in two-liter culture LB medium with antibiotics overnight. Cells were spun down and DNA was extracted using standard alkaline-lysis method. DNA was dissolved in double distilled water and Cesium chloride (CsCl) was added at a 1 g/1 ml ratio. The solution was warmed up to 30°C to facilitate the dissolution of the salt. Eight milliliters of a solution of ethidium bromide (10 mg/ml in water) were added to every 100 ml of the DNA/CsCl salutation. The ethidium bromide solution was immediately mixed with the denser DNA/CsCl solution. The final density of the solution should be 1.55 g/ml, and the concentration of ethidium bromide should be approximately 740 μg/ml.
DNA/CsCl solution was added into 15 ml high-speed Quick-Seal tubes (16 x 76 mm, Beckman, Palo Alto, CA) and centrifuged at 75,000 rpm for 16 hours at 20°C in a XL-90 Ultracentrifuge (Beckman, Palo Alto, CA) rotor. Three layers were formed and the clear, red one in the middle contained the DNA. An 18-gauge needle was used to transfer the clear red solution into another 15 ml tube, and CsCl was added again and DNA-CsCl solution was centrifuged again to obtain purified plasmid DNA. The plasmid DNA band was removed and transferred into a 50 ml plastic tube. Aluminum foil was used during the whole procedure to avoid DNA degradation by light.

Isobutanol saturated with water was then used to remove the ethidium bromide from the DNA solution. An equal volume of isobutanol was added to the DNA solution and mixed well by vortexing. The upper layer of solution containing ethidium bromide was removed. The procedure was repeated at least 10 times until all the pink color disappeared from both the aqueous phase and the organic phase.

CsCl was removed from DNA solution by dialysis through Slide-A-Lyzer 2K™ dialysis Cassettes (Pierce, Rockford, IL, USA). The solution was dialysed twice in two liters TE buffer for 24 hours at 4°C. DNA was finally precipitated with 10% Ammonium Acetate and 3 volumes of 100% ethanol for five hours at −20°C followed by centrifugation at 10,000 x g for 30 minutes at 4°C. DNA was resuspended in double distilled water at a concentration of 4 μg/μl.
I. Preparation of *E.coli* competent cells

Media (SOB, SOC, and LB) were prepared as described by Hanahna (Hanahan, *et al.*, 1983). SOC medium is the same as SOB medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, and 10 mM MgSO₄) except for the adding of 20 mM glucose. For selection of transformed *E.coli*, LB plates containing 100 μg/ml ampicillin were used. To make Transfer Buffer (TB) (10 mM Pipes, 55 mM MnCl₂, 15 mM CaCl₂, and 250 mM KCl), all the components except for MnCl₂ were mixed and the pH was adjusted to 6.7 with KOH. Then MnCl₂ was dissolved, the solution was sterilized by filtration through a 0.45 μm filter unit and stored at 4°C. All salts were added as solids.

Frozen stock of DH5α cells were thawed, streaked on to an ampicillin/LB agar plate, and cultured overnight at 37°C. Ten to twelve large (2-3 nm in diameter) colonies were isolated with a plastic loop, inoculated into 250 ml of SOB medium in a 2-liter flask, and grown to an OD₆₀₀ of 0.6 at 18°C with vigorous shaking (200-250 rpm). The flask was removed from the incubator and placed on ice for 10 minutes. The culture was transferred to a 500 ml centrifuge bottle and spun at 2500 x g for 10 minutes at 4°C. The pellet was resuspended in 80 ml of ice-cold TB, incubated in an ice bath for 10 minutes, and spun down as described above. The cell pellet was gently resuspended in 10 ml of TB, and DMSO was added with gentle swirling to a final concentration of 7%. After incubating in an ice bath for one minute, the cell suspension was dispensed by 1-2 ml into tissue-culture cell-freezing tubes and immediately chilled by immersion in liquid
nitrogen. The frozen competent cells were stored at -70°C for at least 4 months without a detectable loss of competence.

**J. Polymerase Chain Reaction (PCR)**

The PCR reaction was conducted as previously described (Sambrook *et al.*, 1989). The PCR amplification mixture was prepared by adding reagents to a sterile microcentrifuge tube in the following order: 10 μl of 10 x Reaction Buffer (250 mM MgCl₂, 100 mM Tris, 500 mM KCl, pH8.3), 2 μl of 10 mM PCR nucleotide Mix, 5 μl of 20 μM upstream and downstream primers (1 μM final concentration), 1 μl of template DNA (50 μg/μl), 1 μl of DNA polymerase (5 U/μl), and H₂O were added to bring final volume to 100 μl.

The condition of PCR for amplification of normal length DNA fragments (from 200 bp to 2.5 kb), PCR was performed using *pwo* polymerase (Boehringer Mannheim) under the following conditions: 94°C for 2 min for one cycle, followed by 94°C for 30 sec, +55°C for 30 sec, +65°C for 60 sec for 25 cycles.

The condition of PCR for IL-2 leader sequence was as described previously: PCR was performed using *pwo* polymerase (Boehringer Mannheim) under the following conditions: 94°C for 2 min for one cycle, followed by 94°C for 15 sec +50°C for 30 sec for 30 cycles.
K. Site-directed mutagenesis

The Quick Change™ Site-Directed Mutagenesis Kit from Stratagene was used to introduce mutation in the *Renilla luciferase* gene. Plasmid pND2-SRUC was used as a template and two primers were designed for one amino acid substitution. Sample reactions were prepared as described: 5 µl of 10 x reaction buffer, 25 ng of ds DNA template, 125 ng of oligonucleotide A, 125 ng of oligonucleotide B, 1 µl of dNTP mix, and H₂O was used to bring a final volume to 50 µl. One µl of *Pfu* polymerase (2.5 U/µl, Stratagene, San Diego, CA) was added right before the cycling reaction.

Cycling reactions were performed using *Pfu* DNA polymerase under the following condition: 95°C for 30 seconds for one cycle, followed by 95°C for 30 sec, 55°C for one min, 68°C for 10 min, 30 sec for 16 cycles. Following temperature cycling, the reactions were cooled on ice for 2 minutes. Ten Units of *Dpn I* restriction enzyme were directly added into each amplification reaction to digest parental DNA template at 37°C for one hour. The amplified DNA was then used to transform *E.coli* XL1-Blue competent cells. Three colonies were randomly selected for DNA sequencing analysis.
L. Western blotting analysis

Western blotting analysis was conducted as previously described (Sambrook et al., 1989). Transfected mammalian cells were harvested 48 hours after transfection by scraping with a rubber policeman 100 µl 2 x gel-loading buffer (0.1% Bromophenol Blue, 1M sodium phosphate, pH7.4, 4% SDS, 20% glycerol) and transferred into a 1.7 ml eppendorf tube. Cells were lysed by sonication for 3 x 10 sec, boiled for 10 minutes, and the supernatants were transferred into another tube. Twenty microliters of sample was loaded on SDS-polyacrylamide mini-gel. Proteins were separated by electrophoresis at 165 volts for 1.5 hours. Protein bands were electrotransfered to PVDF membrane for 2 hours by a LABCONCO semi-dry blotter.

The membrane was then washed with 1 x TBST (20 mM Tris-HCl; 137 mM Sodium Chloride; 3.8 mM Hydrochloric acid; 0.1% Tween-20), and then was blocked with 1% Gelatin/ TBST at room temperature overnight. The membrane was washed three times (1x15 min, and 2x5 min) with TBST, and incubated with antibodies (1:10,000 dilution in TBST) raised against the protein of interest for two hours at room temperature on a shaker. After being washed three times, the membrane was incubated with anti-mouse IgG2a conjugated to Peroxidase (Amersham) diluted 1:15,000 in TBST for one hour at room temperature before being washed with TBST buffer. The blotted membrane was incubated with SuperSignal Substrate Working Solution (Pierce, Rockford, IL, U.S.A.) for 5 minutes. The working solution was removed and blot was
wrapped in plastic warp. A Kodak X-ray film was exposed to the blot for chemiluminescent detection and the film was developed.

**M. Protein labeling with \(^{35}\text{S}\)-methionine**

To detect secreted GAD proteins in cell culture media, \(^{35}\text{S}\)-methionine (specific activity >1000 Ci/mmol, from DuPont NEN, Boston, MA) was used to label total cell protein from COS-7 cells. Media was removed after incubation with the DNA-calcium phosphate complex, and cells were rinsed twice with 1 x PBS and once with medium without methionine and serum. Cells were then incubated in 3 mL DMEM medium without methionine + 1% dialyzed FBS for 20 min to deplete intracellular pools of endogenous methionine. One hundred microcuries of \(^{35}\text{S}\)-methionine were then added directly into media for protein labeling. Cells were incubated for 24 hours before being harvested.

**N. Immunoprecipitation of secreted proteins**

The Immunoprecipitation Kit (Protein A) from Boehringer Mannheim was used in the experiment. Thirty-six hours after transfection, cells were washed with 1x PBS twice. One hundred microliters of cooled lysed buffer (50 mM Tris-HCL; 150 mM NaCl; 1% Nonidet P40, 0.5% sodium deoxycholate; 1 Complete\textsuperscript{TM} protease inhibitor cocktail tablets, pH 7.5) were added into plates and cells were scraped into an Eppendorf tube
with a rubber policeman. Cells were lysed by sonicating 3 x 10 seconds. Homogenized suspension was centrifuged at 12,000 x g 10 minutes at 4°C to remove cell debris.

To immunoprecipitate secreted protein from cell culture medium, medium was collected 36 hours after transfection, centrifuged at 800x g at 4°C to remove dead cells and cell debris, and Centriprep-10 concentrators (Amicon, CA) were used to concentrate medium from 2.5 ml to 0.5 ml. 2 x lysis buffer (100 mM Tris-HCl; 300 mM NaCl; 2% Nonidet P40, 1% sodium deoxycholate, pH 7.5); 2 Complete™ protease inhibitor cocktail tablets was mixed with concentrated medium at 1:1 ratio.

To reduce background caused by nonspecific binding of irrelevant cellular proteins to protein A/G-agarose, a precleaning step was performed: 50 µl of the homogeneous protein A/G-agarose suspension (25 µl bed volume) was added to the sample and incubated for at least 3 hours at 4°C on a rocking platform. Beads were then pelleted by centrifuged at 12,000x g for 20 seconds, and the supernatant was transferred to fresh tubes.

To immunoprecipitate the target proteins, the appropriate amount of the specific monoclonal antibody (5 µl) was added to samples and gently shaken for 2 hours at 4°C. Fifty microliters of the homogeneous protein A/G-agarose suspension (25 microliters bed volume) were added into the mixture and incubated at 4°C overnight. Complexes were collected by centrifugation at 12,000 x g for 20 s. The supernatant was removed and beads were resuspended in 1ml lysis buffer and incubated at 4°C for another 20 minutes on a rocking platform. The beads were washed with 1 ml lysis buffer one more time.
Beads were collected and washed with 1 ml wash buffer 2 (50 mM Tris-HCl; 500 mM NaCl; 1% Nonidet P40, 0.05% sodium deoxycholate, pH 7.5) twice before being washed with wash buffer 3 (50 mM Tris-HCl; 1% Nonidet P40, 0.05% sodium deoxycholate, pH 7.5). Beads were then centrifuged at 12,000 x g at 4°C and the supernatant was removed.

Fifty microliters of gel-loading buffer were added to the agarose pellet. Proteins were denatured by heating in boiling water for 3 min. The protein A/G- agarose was removed by centrifuged at 12,000 x g for 20 seconds at room temperature, and the supernatant was transferred to fresh tubes. The immunoprecipitates were fractionated by SDS-polyacrylamide gel electrophoresis.

O. Chemiluminescent SEAP assay

The secreted form of human placental alkaline phosphatase (SEAP) has been used as a reporter molecule to monitor the activity of promoters and enhancers. The chemiluminescent SEAP assay was performed following the User Manual from Clontech, Palo Alto, CA.

Great EscAPe SEAP™ Assay Kit was purchased from Clontech and performed as described. One ml 1X lysis buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM MgCl₂, and 0.1% Triton X-100) was used to homogenize muscle samples. Fifty microliters homogenized tissue and 50 µl serum samples were used in each assay. Tissue and serum samples were mixed with 75 µl 1 x dilution buffer and incubated at 65°C for 30 minutes.
Samples were cooled to room temperature by being placed on ice for 2-3 minutes, and then being equilibrated to room temperature. 100 µl of assay buffer was added to each sample and incubated for 5 minutes at room temperature. One hundred µl of 1.25 mM chemiluminescent substrate CSPD (CSPD: chemiluminescent =1:19) was added to each sample and incubated for 30 minutes at room temperature before light production was measured in TURNER TD-20e luminometer.

P. Intramuscular DNA injection

The protocols for animal experiments were approved by the animal care committee at Loma Linda University. Seven-week-old BALB/c mice (about 25 gm body weight, Jaskson Lab) and three-week-old NOD (about 15 gm body weight, Taconic Lab) were maintained in Loma Linda University animal care facility during the experiment. Mice were anesthetized by i.p. injection with Ketamine (66 mg/Kg body weight, Phoenix Pharmaceutical, St. Joseph, M), xylazine (7.5 mg/Kg body weight, LLOYD Laboratories, Shenandoah, Iowa), and Acepromazine Maleate (1.5 mg/Kg body weight, Fermenta, Kansas City, MO. Plasmid DNA (200 µg/leg) was injected into mice quadriceps muscle with a 1-cc sterile 27G1/2 syringe (Becton Dickinson Company, Franklin Lakes, NJ). One hundred microliter (200 µg) of DNA were injected into each leg. NOD mice received a second injection five days after the first one in a similar fashion.
Q. Mitogen stimulation assay for *GAD65*

About $5 \times 10^6$ spleen cells were isolated from NOD mice. Isolated lymphocytes were cultured with GAD65 purified protein for 72 hours, at which time the maximal effect of DNA synthesis should be seen. The measurement of this DNA synthesis was accomplished by pulsing the cultures with tritiated thymidine, a nucleotide precursor that is incorporated into DNA. The lymphocytes are then harvested and later counted on a scintillation counter. The counts obtained quantitate DNA synthesis, and represent lymphocyte responsiveness to the nonspecific antigens. The data are expressed as counts from the stimulated cultures divided by counts from unstimulated cultures, which yields a ratio, called the Stimulation Index.

R. Histopathological analysis of insulitis

Ten-week-old NOD mice were killed and the pancreatic islets were removed for insulitis scoring. The pancreas were placed into Bouin’ fixative (75 ml picric acid, 25 ml formalin, 5 ml glacial acetic acid) for two days. Pancreas samples were dehydrated after being placed into an increasing concentration of alcohol (30, 50, 70, 80, 95% and 100% alcohol) until disappearance of yellow color. Each stain required two to three hours. Pancreas were incubated in 100% alcohol one more time to remove water completely.
Paraffin was melted at 56 to 58 °C. Tissues were placed in small container filled with melted Paraffin and were cooled in water. The microtome was used for slide preparation. The average thickness of each section was 5 microns. The slides were stained with hematoxylin, and then counterstained with eosin.

Average of fifteen islets per mouse were scored. A 7-level semi-quantitative scoring scale (Zhang et al., 1991) was used for insulitis scoring: 0: normal islet tissue without any detectable lymphocytes infiltration; 1: focal peri-islet T cell infiltration with less than one-third of the peri-islet area; 2: more extensive peri-islet T cell infiltration with less than two-thirds of the peri-islet area; 3: peri-islet T cell infiltration with more than two-thirds of the peri-islet area; 4: intra-islet T cell infiltration with less than one-third of the islet area; 5: intra-islet T cell infiltration with less than two-thirds of the islet area; 6: severe intra-islet T cell infiltration with more than two-thirds of the islet area.

Scoring of 1-3 indicated peri-insulitis, and scoring of 4-6 indicated intra-insulitis. Scoring was performed using the double-blind method by two different scorers.
CHAPTER THREE
SECRETION OF FUNCTIONAL RENILLA RENIFORMIS
LUCIFERASE BY MAMMALIAN CELLS

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¹ Graduate student who conducted all the experiments
² Professor at the department of Laboratory Medicine and Pathology, Rochester, Minnesota who provided the cDNA of Renilla luciferase, Renilla luciferase purified protein, anti-Renilla luciferase antibody, and luciferin (substrate for Renilla luciferase)
³ Student’s mentor and chairman of research committee who directed research and supervised the manuscript and dissertation

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Secretion of functional *Renilla reniformis* luciferase by mammalian cells

(Recombinant DNA; reporter gene; eukaryotic gene expression; bioluminescence)

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Abbreviations: aa: amino acid(s); AP: alkaline phosphates; bp: base pair(s); CMV: cytomegalovirus; FBS: fetal bovine serum; GFP: green fluorescent protein; IL: interleukin; IL2SP: interleukin-2 signal peptide; KDa: kilodalton(s); Mr: molecular weight; PAGE: polyacrylamide gel electrophoresis; PBS: phosphate buffer saline; PCR: polymerase chain reaction; RUC: *Renilla reniformis* luciferase.

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1. ABSTRACT

The soft coral *Renilla reniformis* luciferase enzyme (RUC) is a monomeric soluble intracellular protein that is increasingly being used as a marker of gene expression. Here the *ruc* gene was engineered to encode a protein product secreted by mammalian cells. The 5' end of the *ruc* gene was fused in frame with the 3' end of a short DNA sequence encoding the signal peptide from human interleukin-2 (IL-2) protein. This construct was cloned under the transcriptional control of the cytomegalovirus (CMV) promoter into a mammalian expression vector. Simian COS-7 cells were transiently transfected with the construct, and light emission was measured both from cell lysates and from cell culture media. Results indicated that RUC was secreted as a functional protein by mammalian cells. The advantages and disadvantages of secreted RUC as a marker of gene expression when compared to other secreted protein markers are discussed.
2. INTRODUCTION

Light emission from bioluminescence or chemiluminescence has become a powerful tool in molecular biology for detecting the presence of certain molecules as well as for studying specific cellular events. Marker genes encoding luciferases provide rapid and sensitive means of detecting gene expression in a quantitative manner both \textit{in vitro} and \textit{in vivo}. The most widely used luciferases for this purpose are from the bioluminescent \textit{Vibrio} bacteria, from the jellyfish \textit{Aequoria victoria}, and from the firefly \textit{Photinus pyralis}. Recently the use of the \textit{A. victoria} green fluorescent protein (GFP), a photonprotein, has permitted the direct visualization of the location of various proteins within a living cell, and of the movement of living organisms within their environment. However none of the above reporter proteins is considered universal in its application, and the use of other less well known luciferases or GFPs could be better suited for particular experimental conditions. The luciferase from \textit{Renilla reniformis} is an example of such a reporter protein.

The sea pansy \textit{Renilla reniformis} is an anthozoan coelenterate living in shallow coastal waters of North America, and can display blue-green bioluminescence (Matthews, \textit{et al.}, 1977). The light emission activity is catalyzed by a luciferase enzyme. The monomeric RUC protein (M\textsubscript{r}: 36 kDa) catalyzes the oxidative decarboxylation of the complex organic molecule coelenterazine (the luciferin) in the presence of dissolved oxygen to yield oxyluciferin, CO\textsubscript{2}, and blue light (\(\lambda\text{max}=480\text{nm}\)) \textit{in vitro}. In \textit{R. reniformis}, energy transfer from the luciferase-bound oxyluciferin excited state donor
to a green fluorescent protein acceptor results in blue-green light emission
($\lambda_{\text{max}} = 509$ nm). A cDNA encoding a functional full-length RUC protein was
previously cloned and sequenced (Lorenz et al., 1991). This cDNA has been used as a
marker of gene expression in bacterial (Lorenz et al., 1991), plant (Mayerhofer et al.,
1995), yeast (Shrikanta et al., 1996), and mammalian cells (Lorenz et al., 1996).

The firefly luciferase gene is the most often used luciferase marker gene in
eukaryotic cells. When compared in vitro, the specific quantum yield of RUC is
approximately one order of magnitude less than that of firefly luciferase (Sherf et al.,
1996). However in some cases in vivo, cells expressing the ruc gene show much higher
levels of light emission than the same cells expressing the firefly luciferase gene. For
instance in the case of the yeast Candida albicans, cells expressing the firefly luciferase
gene show little bioluminescence activity (Shrikanta et al., 1995). This absence of
activity is probably due to the synthesis of a non-functional firefly luciferase enzyme,
since the firefly luciferase gene contains nine CUG codons which are translated to serine
in C. albicans, instead of the conventional leucine (Santos et al., 1993). Because the ruc
gene does not contain any CUG codons, it encodes a fully functional protein in this
organism (Shrikanta et al., 1996). Plants are another example of the occasional
superiority of the ruc gene as a marker over other luciferase genes: light emission
obtained from electroplated alfalfa protoplasts and transgenic tobacco, tomato, and potato
plants is much higher when the ruc gene is used instead of the bacterial or firefly
luciferase genes (Mayerhofer et al., 1995).
In the work presented here, the range of application of the *ruc* gene as a marker of gene expression has been extended by fusing it in frame with a DNA fragment encoding the human IL-2 signal peptide (IL2SP). When expressed in mammalian cells this construct encodes a product that is secreted as a functional RUC enzyme. This new reporter gene could be best suited for the monitoring of gene expression in animal models.

3. MATERIALS AND METHODS

3.1 Plasmids construction

pLNCX-RUC: plasmid pLNCX-RUC was constructed by ligating the *ruc* gene as a 975 bp EcoRV-Smal DNA fragment from plasmid pBluescript II KS(+) carrying the *ruc* gene (Mayerhofer et al., 1995) into the Hpal site of plasmid pLNCX (Miller and Rosman, 1989), generating plasmid pLNCX-RUC (Fig.1A).

pLNCX-ILRUC: an 87 bp DNA fragment encoding the first 23 amino acid residues of IL-2 protein that contain IL2SP was amplified from human embryonic kidney cell line A293 genomic DNA using the polymerase chain reaction (PCR) with primers IL-01 (5' TTTGAATTCCATGTACAGGATGCAACTCCT3') and IL-02 (5' TTTGAATTCCATGTACAGGATGCAACTCCT3'). PCR was performed using Pwo polymerase (Boehringer Mannheim) under the following conditions: 94°C for 2 min for one cycle, followed by 94°C for 15 sec + 50°C for 30 sec for 30 cycles. The PCR fragment was cloned directly into vector pGEMT (Promega), excised using the EcoRI
sites introduced by both primers, and ligated 9 bp upstream of the *ruc* gene in plasmid pLXSN-RUC (the construction of pLXSN-RUC will be described elsewhere). The correct sequences of the PCR product and of the in-frame fusion of this product with the *ruc* gene were confirmed by automated DNA sequencing. The DNA sequence encoding the IL2SP-RUC protein fusion was amplified using primers IL-01 and RUC-1 (5' TTTCCCGGAAAAATGTAAATAAAAAACCA3') and cloned into plasmid pGEMT. The PCR product was then subcloned as a *KspI-SmaI* fragment into the *HpaI* site of plasmid vector pLNCX, generating plasmid pLNCX-ILRUC (Fig. 1B).

3.2 Cell culture and transfection

Simian COS-7 cells were grown in 60 mm tissue culture dishes containing 3 ml DMEM medium supplemented with 10% fetal bovine serum (FBS). Medium was changed 3 hrs prior to transfection when cells were 75% confluent. Cell transfection was performed according to the ProFection calcium phosphate system (Promega) using 25 μg plasmid DNAs per plate. Cells were washed twice with PBS buffer 6 hrs after transfection, and 3 ml DMEM+10% FBS was added to each plate.

3.3 RUC assay

Cells were harvested by trypsinization 48 hrs after transfection, washed once with RUC assay buffer (0.5 M NaCl, 1mM EDTA, 100mM potassium phosphate, pH 7.4), resuspended in 1 ml of the same buffer, and sonicated on ice twice for 10 sec. Five
hundred microliters of cell lysates was assayed in each case for bioluminescence in a luminometer (Turner TD-20e) after rapid injection of 500 μl 1 μM coelenterazine hcp (Molecular Probes Inc.). Media from transfected cells were saved at harvesting and centrifuged at 800g to remove cell debris. Two hundred microliters aliquots of media were assayed for bioluminescence as described above.

3.4 Time-course assay of secreted RUC

The RUC assay was performed every 12 hrs with 200 ul of media taken from dishes containing growing cells transiently transfected with plasmids pLNCX, pLNCX-RUC, or pLNCX-ILRUC. Two hundred microliters of fresh media was added each time to the plates as replacement for the taken aliquot. With one plate the medium was changed after 24 hrs to serum-free medium (QBSF 51 from Sigma) supplemented with 1% FBS to investigate the effects of medium composition on accumulation of functional secreted RUC.

3.5 Measurement of RUC stability in cell culture media and in blood

Three nanograms of purified RUC isolated from an overexpressing Escherichia coli strain was added to a 10 cm plate containing COS-7 cells growing at 70% confluence in 10 ml DMEM +10% FBS, or in 10 ml QBSF 51+1% FBS. The plates were incubated at 37°C in 5% CO2, and a 200 μl aliquot of medium was taken every two hours for bioluminescence assay. To measure stability of RUC in blood, 3 ng of isolated RUC was
added to 700 µl of complete hamster blood containing 50 µl of a heparin solution (1000 units/ml, from Elkins-Sinn) and placed at 37°C. A twenty microliters aliquot was taken at regular time intervals and diluted into 200 µl RUC assay buffer for bioluminescence assay.

3.6 Immunoblot analysis of RUC

For immunoblot analysis of intracellular RUC, COS-7 cells were washed twice with cold phosphate buffer saline (PBS) buffer 48 hrs after transfection, and harvested by scrapping in 200 µl PBS buffer using a rubber policeman. Cells were pelleted at 10,000g, resuspended into 100 µl 2x gel-loading buffer (100 mM Tric.HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.01% bromophenol blue), and lysed by sonication. Samples were placed in boiling water for 3 min, centrifuged at 10,000g for 10 min to pellet debris, and 30 µl of each sample was loaded on a 12% SDS-polyacrylamide gel. Fractionated proteins were then transferred onto a nylon membrane, reacted with a polyclonal antibody raised against RUC as previously described (Mayerhofer et al., 1995), and detected using chemiluminescence.

For immunoblot analysis of secreted RUC, 1.5 ml aliquots of cell culture media (QBSF 51 + 1% FBS) were taken 48 hrs after transfection of COS-7 cells and centrifuged at 10,000g to pellet cell debris. The aliquots were concentrated down to 0.5 ml using a centriprep concentrator (Amicon) with a molecular weight cut-off of 15 kDa. RUC present in the concentrates was immunoprecipitated using the Protein A
Immunoprecipitation Kit (Boehringer Mannheim) and polyclonal antibodies raised against RUC, and immunoanalyzed after SDS-PAGE using the same antibodies as described above.

4. RESULTS

4.1 Construction of pLNCX-ILRUC

The signal peptide of human IL-2 protein was previously used to cause secretion by mammalian cells of human leukocyte elastase (M; 27 kDa) when fused to its amino terminal domain (Okano et al., 1990). In the present work, the DNA sequence isolated by PCR using primers II-01 and IL-02 encodes the first 23 amino acid residues from human IL-2, with the putative site of cleavage of the signal peptidase situated between residues 20 and 21 (Taniguchi et al., 1983). The Ilruc gene encodes the 23 aa IL-2 signal sequence fused to the RUC protein by an additional 5 aa as a result of the cloning procedure (Fig. 1B). The Ilruc gene was cloned under the transcriptional control of the CMV promoter into retroviral vector pLXSN for expression studies, and for future packaging into retroviral particles and introduction into animal models.

4.2 Expression of the ruc and Ilruc genes in mammalian cells

Plasmids pLNCX, pLNCX-RUC, and pLNCX-ILRUC were introduced into simian COS-7 cells using the calcium phosphate transfection method. No differences in cell morphology or cell growth were observed between cells expressing the ruc gene and
cells expressing the Ilruc gene fusion (data not shown). After 48 hrs, RUC activity was measured both from cell lysates and from the medium (Fig.2). Luciferase activity measured from lysates of cells expressing the wild type ruc gene was approximately 15 times higher than that measured from lysates of cells expressing the Ilruc gene fusion (Fig.2A). In contrast, RUC activity could be measured from cell culture medium only in the case of cells expressing the Ilruc gene (Fig. 2B).

4.3 Time course of appearance of RUC activity in cell culture media

RUC activity could be detected in cell culture medium (DMEM+10% FBS) less than 10 hrs after cell transfection, but only with cells transfected with plasmid pLNCX-ILRUC. In contrast, medium taken from plates containing cells transfected with plasmid pLNCX-RUC did not show detectable RUC activity (Fig.3). In the case of cells transfected with pLNCX-ILRUC, luciferase activity increased over time until it reached a plateau 48 hrs after transfection. Significantly, when cell growth was slowed down by replacing the medium with QBSF 51 medium+1% FBS, RUC activity was still increasing 80 hrs after transfection (Fig.3).

4.4 Stability of RUC in cell culture media and in blood

Isolated RUC was added to plates containing COS-7 cells growing in DMEM medium +10% FBS or in QBSF 51 medium +1% FBS, and bioluminescence activity was measured over time from the media. The half-life of RUC was similar in both media at
37°C (Fig. 4), with calculated values of 50 min and 57 min, respectively. The half-life of isolated RUC in undiluted complete blood at 37°C was calculated to be 36 min.

4.5 Immunodetection of RUC

Presence of RUC both in cell lysates and in cell culture media was investigated using immunoblot analysis after protein fractionation with SDS-PAGE. A protein band corresponding to the RUC protein was found in lysates of COS-7 cells transfected with plasmids pLNCX-RUC and in lysates of cells transfected with pLNCX-ILRUC (Fig. 5A). When cell culture media were analyzed, RUC was found only in the case of cells transfected with pLNCX-ILRUC (Fig 5B).

5. DISCUSSION

Several secreted proteins have been used as markers of gene expression in recent years. The main advantage of using a secreted reporter protein versus an intracellular reporter protein is the ability to measure gene expression without destroying the cells or tissues containing the marker gene. This ability is particularly useful when doing time-course studies of transcriptional activity, for instance in cell cultures (Cullen and Mallim, 1992), during embryo development (Thompson et al., 1995), or when monitoring gene expression \textit{in situ} after introduction of naked DNA or genetically engineered cells in animal models (Naffakh \textit{et al.}, 1996).
The most sensitive secreted reporter proteins are light-catalyzing and light-emitting proteins such as alkaline phosphatase (AP) (Berger et al., 1988), Vargula hilgendorfii luciferase (Thompson et al., 1990), apoaequorin (Inouye and Tsuji, 1992), and GFP from Aequoria victoria (Kaether and Gerdes, 1995; Laukkanen et al., 1996). Of all these proteins only the Vargula luciferase is naturally secreted. However, its substrate is not commercially available, therefore restricting its use as a reporter protein. Also restricted is the use of secreted GFP by mammalian cells, due to a requirement for temporary arrest of protein transport in order to allow formation of the GFP chromophore within the cell (Kaether and Gerdes, 1995). To our knowledge there has been no report of secretion by living cells of active bacterial luciferase or of active firefly luciferase, although both enzymes can recover their function after translocation into microsomes in vitro (Brunke et al., 1996; Tyedmers et al., 1996).

In the work presented here, a DNA sequence encoding the signal peptide of the human IL-2 protein was fused in frame with the open reading frame encoding the RUC protein, generating the Ilruc gene construct. Light emission measured from cell culture media showed that media in which cells expressing the Ilruc gene were growing (ILRUC medium) contained significant levels of RUC activity (Fig.2B, lane3). This result was unlike the result obtained with medium in which cells expressing the ruc gene encoding wild type RUC were growing (RUC medium), which did not contain detectable luciferase activity (Fig.2B, lane2). In addition, immunoblot analysis of ILRUC medium showed the presence of a protein band with a molecular weight corresponding to that of RUC,
together with another protein band of lower molecular weight, likely the result of RUC protein degradation (Fig.5B, lane 2). Immunoblot analysis of RUC medium showed the presence of a faint protein band corresponding to the putative RUC degradation product (Fig. 5B, lane 3). These results indicated that mammalian cells expressing the Ilruc gene construct secreted a functional Renilla luciferase protein. The luciferase activity measured in the ILRUC medium was not a result of cell lysis, since 3 ml of ILRUC medium (DMEM+10% FBS) from a 100% confluent 60 mm culture dish contained on average 375 relative light units (RLU) 48 hrs after transfection, while cell lysates from the same plate contained on average only 60 RLU. Furthermore, when cell growth was slowed down by changing medium to QBSF 51 + 1% FBS, a medium where RUC stability does not change significantly when compared to DMEM+ 10% FBS (Fig.4), the time of accumulation of RUC in the medium could be extended by at least 30 hrs (Fig.3), another indication that RUC protein was secreted.

Light emission measured from cell lysates showed that cells expressing the rue gene contained approximately 15 times more RUC activity than cells expressing the Ilruc gene construct (Fig.2A). However, immunoblot analysis of cell lysates revealed that this increase in activity could not be explained by an identical increase in RUC protein amounts (Fig.5A), indicating that intracellular RUC in cells expressing the Ilruc gene had a lower activity than intracellular RUC in cells expressing the rue gene. The reason for this reduced activity is not clear, but since the RUC protein contains three predicted cysteine residues, it is possible that the reduced activity was due to formation of various
illegitimate disulfide bonds in the endoplasmic reticulum. If this were the case, it could be possible to improve the sensitivity of secreted RUC as a reporter protein by replacing these cysteine residues with other amino acids. The fact that cells expressing the Ilruc gene contained less RUC protein than cells expressing the rue gene was another indication that Ilruc encoded a secreted product, presumably because the RUC protein fused to IL2SP did not accumulate in the cytosol.

To be advantageous a secreted protein marker should allow for a sensitive and rapid assay, and should have a short half-life. The assay for secreted RUC is not as sensitive as the assay for secreted AP, itself 10-fold more sensitive than firefly luciferase in mammalian cells, but it is comparable to the assay for secreted apoaequorin (Inouye and Tsuji, 1992). However, the rapidity of the RUC assay (<1 min) is a significant advantage when compared to the secreted AP assay which requires at least 45 min (Berger et al., 1988), and when compared to the secreted apoaequorin assay which requires at least 2 hrs (Inouye and Tsuji, 1992). Although a short half-life is not absolutely required when previously secreted protein can be removed before the beginning of a time-course study, it becomes an important factor if the study is to be performed in a closed system, such as within an animal. RUC had a half life of 50 min in DMEM + 10 % FBS, 57 min in QBSF 51 + 1% FBS, and 36 min in blood, which should allow monitoring of gene expression in closed systems.

Secreted RUC could be best applied for the monitoring of gene expression in animal models. Measuring activity of a secreted protein marker in mammals permits the
monitoring of gene expression directly in situ without killing the animal. The naturally secreted human growth hormone protein has been used for this purpose (Selden et al., 1986). However, ideally a secreted marker of gene expression in mammals should be "neutral", i.e., it should not have a biological function having an effect on the metabolism and/or the development of the animal, as human growth hormone can have (Hackett and Gitlin, 1992). Thus, although AP seems to be a more sensitive reporter protein than RUC, it might not be the best choice for synthesis in animals because high levels of AP in blood may cause unwanted physiological side-effects, and because of possible elevated background readings due to alkaline phosphatases already present in sera. Secreted RUC could be a good candidate as a marker of gene expression in this case, since RUC bioluminescence activity can be quickly measured from blood, and because we do not foresee a physiological effect of secreted RUC in animals. In addition, background bioluminescence upon injection of coelenterazine in blood was not significant (data not shown), and although undiluted complete blood quenched RUC activity by a factor of ten, this activity could be completely recovered after a ten fold dilution into RUC assay buffer (data not shown).

In summary, secreted RUC is a bioluminescent reporter protein that offers a sensitive, rapid assay, whose substrate is commercially available, and whose activity is unaffected by a variety of media and sera (Ogbonna and O'Kane, 1994). We are presently investigating the use of secreted RUC for monitoring gene transfer and expression in animal models.
ACKNOWLEDGEMENTS

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Figure 1. Partial structures of the plasmids used in this study. Ampicillin resistance gene and bacterial origin of replication are not shown. A. Plasmid pLNCX-RUC carries a cDNA encoding the wild-type RUC protein. B. Plasmid pLNCX-ILRUC carries the Ilruc gene encoding IL2SP fused to the wild type RUC protein; the aa sequence of the protein fusion junction is shown: aa14-23, C-end of IL2SP; aa24-28, linker aa; aa29, N-end of RUC; the arrow indicates the putative cleavage site for signal peptidase. LTR, long terminal repeat; neo, neomycin resistance gene; pA, polyadenylation site.
A. pLNCX-RUC

\begin{center}
\begin{tikzpicture}
  \node[rectangle, draw] (LTR) at (0,0) {LTR};
  \node[rectangle, draw] (neo) at (1,0) {neo};
  \node[rectangle, draw] (CMV) at (2,0) {CMV};
  \node[rectangle, draw] (rue) at (3,0) {rue};
  \node[rectangle, draw] (LTR2) at (4,0) {LTR};
  \node[rectangle, draw] (pA) at (4,0.5) {pA};
  \draw[->] (LTR) -- (neo);
  \draw[->] (neo) -- (CMV);
  \draw[->] (CMV) -- (rue);
  \draw[->] (rue) -- (LTR2);
  \draw[->] (LTR) -- (pA);
  \draw[->] (LTR2) -- (pA);
\end{tikzpicture}
\end{center}

B. pLNCX-ILRUC

\begin{center}
\begin{tikzpicture}
  \node[rectangle, draw] (LTR) at (0,0) {LTR};
  \node[rectangle, draw] (neo) at (1,0) {neo};
  \node[rectangle, draw] (CMV) at (2,0) {CMV};
  \node[rectangle, draw] (luc) at (3,0) {Ilrue};
  \node[rectangle, draw] (LTR2) at (4,0) {LTR};
  \node[rectangle, draw] (pA) at (4,0.5) {pA};
  \draw[->] (LTR) -- (neo);
  \draw[->] (neo) -- (CMV);
  \draw[->] (CMV) -- (luc);
  \draw[->] (luc) -- (LTR2);
  \draw[->] (LTR) -- (pA);
  \draw[->] (LTR2) -- (pA);
\end{tikzpicture}
\end{center}

CTT GCA CTT GTC ACA AAC AGT GCA CCT ACT GAA TTC AGC TTA AAG ATG
Leu  Ala  Leu  Val  Thr  Asn  Ser  Ala  Pro  Thr  Glu  Phe  Ser  Leu  Lys  Met
14   15   16   17   18   19   20   21   22   23   24   25   26   27   28   29
Figure 2. *Renilla* luciferase activity in cell lysates (A) and in cell culture medium (B). Light emission was measured with COS-7 cells transfected with 1, pLNCX; 2, pLNCX-RUC; and 3, pLNCX-ILRUC. Results obtained from six individual transfection are shown. Relative light units (RLU) are shown as per second, per 50µg total protein in (A), and as per second, per 200 µl medium in (B).
Figure 3. Time-course of Renilla luciferase activity appearance in cell culture media. Simian COS-7 cells were transfected with plasmid pLNCX-ILRUC and grown in DMEM medium + 10% FBS (●); transfected with plasmid pLNCX-ILRUC and grown in DMEM medium + 10% FBS for the first 42 hours, and then grown in QBSF 51 medium + 1% FBS (▲); transfected with plasmid pLNCX-RUC and grown in DMEM medium + 10% FBS (♦); and transfected with plasmid pLNCX and grown in DMEM medium + 10% FBS (■). Relative light units (RLU) are shown as per second, per 200 ul medium in (B).
Figure 4. Stability of Renilla luciferase in various media at 37°C. Isolated Renilla luciferase lacking IL2SP was added to DMEM medium + 10% FBS (●), QBSF medium + 1% FBS (♦), and blood (▲), and Renilla luciferase activity was measured over time. Renilla luciferase activity was measured over time as relative light units (RLU), and is shown as per second.
Figure 5. Immunoblot analysis of cell lysates (A) and immunoprecipitated cell culture media (B) using *Renilla* luciferase mAb. Simian COS-7 cells were transfected with plasmid pLNCX-ILRUC (lane 2), plasmid pLNCX-RUC (lane 3), and vector only (lane 4). Lane 1 contains 10 ng isolated *Renilla* luciferase as control. For total cell lysates (A), protein amounts were determined by densitometry after comparison with isolated *Renilla* luciferase protein (lane 1), and calculated to be 0.9 ng for the luciferase encoded by *Ilruc* (lane 2), and 1.7 ng for the luciferase encoded by the *Renilla* luciferase cDNA (lane 3).
CHAPTER FOUR

ENGINEERING OF A SECRETED *RENILLA* LUCIFERASE WITH INCREASED STABILITY

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² Student’s mentor and chairman of research committee who directed research and supervised the manuscript and dissertation

This manuscript has been submitted to *GENE.*
Engineering of a secreted Renilla luciferase with increased stability

(Eukaryotic gene expression; reporter gene; site-directed mutagenesis; disulfide bond)

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Abbreviations: aa: amino acid(s); SEAP: secreted alkaline phosphates; bp: base pair(s); CMV: cytomegalovirus; FBS: fetal bovine serum; KDa: kilodalton(s); M₀: molecular weight; PAGE: polyacrylamide gel electrophoresis; PCR: polymerase chain reaction; SDS: sodium dodecyl sulfate; SRUC: secreted Renilla reniformis luciferase.

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Abstract

We have previously reported the construction of a functional Renilla luciferase enzyme secreted by mammalian cells when fused to the leader peptide of human interleukin-2. The presence of three predicted cysteine residues in the amino acid sequence of Renilla luciferase suggested that its secreted form(s) contained an oxidized sulfhydryl group, which might impair enzyme activity and/or stability. Four secreted Renilla luciferases were constructed to investigate this possibility: three luciferases where in which a different cysteine residue was replaced by an alanine residue, and one luciferase mutant which all three cysteine residues was replaced by alanine residues. Simian cells were transfected with the genes encoding these mutant luciferases, as well as with the original gene construct, and cell culture media were assayed for bioluminescence activity. Only media containing a mutated luciferase with a cysteine to alanine substitution at position 152 in the preprotein showed a marked increase in bioluminescence activity when compared to media containing the original secreted Renilla luciferase. This increase in light emission was due mostly to enhanced stability of the mutant enzyme. This new enzyme represents a significant improvement in the sensitivity of the secreted Renilla luciferase assay.
1. Introduction

Secreted reporter proteins offer the distinct advantage of permitting the monitoring of gene expression over time, without cell or tissue destruction. Examples of proteins used for this purpose are human growth hormone (Selden et al., 1986), and secreted alkaline phosphatase (SEAP) (Berger et al., 1988). Light-emitting reporter proteins such as SEAP are particularly convenient, because their assays offer great sensitivity, and permit easy quantification of the reporter protein (Yang et al., 1997).

We have already described the construction and use of a secreted form of the Renilla luciferase by mammalian cells (Liu et al., 1997). The wild type luciferase enzyme of the sea pansy Renilla reniformis is a monomeric protein with molecular weight 36 kDa, and is normally found intracellularly. This enzyme catalyzes the emission of visible light in presence of oxygen and the luciferin coelenterazine, or one of its analogues (Inouye and Shimomura, 1997), yielding oxyluciferin and carbon dioxide. A cDNA encoding Renilla luciferase has been used to monitor gene expression in bacterial (Jubin and Murray, 1998), yeast (Srikantha et al., 1996), plant (Mayerhofer et al., 1995), and mammalian (Lorenz et al., 1996) cells.

Secreted Renilla luciferase catalyzes a “glow type” of light emission; moreover, its assay can be performed quickly after obtaining samples, and its luciferin is commercially available. Altogether, these properties make secreted Renilla luciferase a potentially attractive marker protein for studying gene expression, or for experimental control in conjunction with other secreted marker proteins. Although naturally secreted
luciferases do exist, for example, the luciferase from the marine ostracod *Vargula hilgendorfii* (Thompson *et al.*, 1989), or from the decapod shrimp *Oplophorus* (Shimomura *et al.*, 1978), their use as practical reporter proteins is still hampered by the fact that either the luciferin is not commercially available, or the encoding gene has not been isolated.

During our studies of secreted *Renilla* luciferase, we have sometimes observed its intracellular aggregation in transfected mammalian cells, depending on undefined cell growth conditions (J.L. and A.E., unpublished data). Because the wild-type *Renilla* luciferase protein sequence contains three predicted cysteine residues (Lorenz *et al.*, 1991), this phenomenon suggested a conformational instability of the secreted *Renilla* luciferase, possibly due to the odd number of cysteine residues present in *Renilla* luciferase. Cysteine residues in a naturally secreted protein are generally found as cystine or buried within the protein, because of the highly reactive nature of the oxidized sulfhydryl group found on unpaired cysteines. Because secreted *Renilla* luciferase presumably folds in the endoplasmic reticulum where sulfhydryl groups are oxidized, the third predicted cysteine residue in its sequence might have interfered with productive protein folding or stability. Therefore, we investigated whether a secreted *Renilla* luciferase with improved bioluminescence activity could be obtained by substituting each of its cysteine residues with alanine, an amino acid considered to be one of the most "neutral" when used for this purpose (Cunningham and Wells, 1989).
In this work, we report the construction and \textit{in vivo} activity of four new secreted \textit{Renilla} luciferases obtained after site-specific mutagenesis of the original secreted \textit{Renilla} luciferase gene construct. One of the mutant luciferases displayed a significant increase in stability, which resulted into up to a 100-fold increase in light emission 48 h after cell transfection when compared to the original secreted \textit{Renilla} luciferase.

2. Materials and Methods

2.1. Plasmid construction and site-specific mutagenesis

The gene encoding secreted \textit{Renilla} luciferase (previously named \textit{Il-ruc} and renamed \textit{sruc} in this report) was excised from plasmid pLNCX-ILRUC (Liu et al., 1997) as a 1.1 kb \textit{HindIII-ClaI} DNA fragment with the \textit{ClaI} site blunt-ended with Klenow enzyme, and was ligated into the \textit{HindIII-SmaI} sites of plasmid pBluescript II KS(+) (Stratagene, San Diego, CA). This gene was then subcloned as a \textit{SalI-XbaI} DNA fragment into plasmid vector pND2 (Gary Rhodes and Robert Malone, unpublished data), under the transcriptional control of the cytomegalovirus promoter, generating plasmid pND2-SRUC.

Plasmid pND2-SRUC then served as a template for site-directed mutagenesis. The Quick Change Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA) was used to introduce three individual cysteine to alanine substitutions at positions 52, 101, and 152 in the preprotein form of secreted \textit{Renilla} luciferase, generating mutant proteins SRUC1, 2, and 3, respectively (Fig.1).
Mutagenesis was performed according to the manufacturer’s protocol, using the following DNA oligomers: SDMRUCA1 (5’CCGCAGTGGTGGGCCAGAGCTAAACAAATGAATGTTCTT3’) and SDMRUCA2 (5’CAAGAACATTCAATTTGTTTAGCTCTGGCCACCACACTGCG3’) for mutagenesis at position 52; SDMRUCB1 (5’GAGCCAGTAGCGCGGGCTATTATACCAGATCTTATTGG3’) and SDMRUCB2 (5’CCAATAAGATCTGGTATAATAGCCCGGCTACTGGCTC3’) for mutagenesis at position 101; and SDMRUCC1 (5’GAGCCATGATTGGGGTGCTGGCTTTGGCATTTCATTATAGC3’) and SDMRUCC2 (5’GCTATAATGAAATGCAAAGGCAGCACCCCCCATCATTGCG3’) for mutagenesis at position 152. In addition, oligomer pairs SDMRUCB1 and B2, and C1 and C2, were used in succession on plasmid pND2-SRUC1 to generate plasmid pND2-SRUC4. This plasmid carries a gene encoding a mutant secreted Renilla luciferase where all predicted cysteine residues in the luciferase were replaced by the amino acid alanine (Fig.1). For mutagenesis, the amplification reaction was performed using a Perkin-Elmer Gene-Amp 2400 using the following program: 95°C for 30 s for one cycle, followed by 95°C for 30 s + 55°C for 1 min + 68°C for 10.5 min for 16 cycles. All mutants were identified by DNA sequencing at the site of mutation. In each case, at least two independent mutants were used for mammalian cell transfection to determine whether additional mutation(s) had been introduced that could affect bioluminescence activity.
2.2 *Cell culture and DNA transfection*

Simian COS-7 cells were grown in 100 mm tissue culture plates containing 10 ml DMEM medium (Sigma Chemical, St. Louis, MO) +10% fetal bovine serum (FBS, Biowhittaker, Walkersville, MD), or in Cellgro complete serum free medium (Mediatech, Herndon, VA) + 1% FBS. Cell transfection was performed using the ProFection Calcium Phosphate System (Promega, Madison, WI), according to the manufacturer’s protocol, using a total of 30 µg of plasmid DNA per plate (25 µg of *Renilla* luciferase plasmid for experimental purpose, and 5 µg of firefly luciferase plasmid for normalization between experiments). Cells were washed twice with phosphate-buffered saline (PBS) 15 h after transfection, and 10 ml of DMEM medium + 10% FBS or Cellgro complete serum free medium + 1% FBS was added.

2.3. *Luciferase assay*

The *Renilla* luciferase assay was performed as previously described (Liu et al., 1997) with a Turner TD-20e luminometer. Unmodified coelenterazine was used as a substrate (Molecular Probes, Eugene, OR, catalog # C-2944). The firefly luciferase assay was performed according to a protocol from the manufacturer of the assay system (Promega, Madison WI). For time course measurement of secreted *Renilla* luciferase activities, simian COS-7 cells were transiently transfected with the different plasmid constructs encoding the secreted *Renilla* luciferases shown in figure 1. Cells were then grown in Cellgro complete serum free medium + 1% FBS for 90 h, and the *Renilla* luciferase assay was performed at regular time intervals with media aliquots.
2.4. Measurement of secreted Renilla luciferase stability in cell culture medium

Stability of the secreted Renilla luciferases showing highest light emission (example, SRUC and SRUC3) was measured in cell culture medium. COS-7 cells grown in 100 mm tissue culture dishes were transiently transfected with 30 μg DNA of plasmid pND2-SRUC and pND2-SRUC3. Thirty-six hours after transfection, 10 ml medium from a dish containing cells transfected with pND2-SRUC, and 200 μl of medium from a dish containing cells transfected with pND2-SRUC3 were taken, centrifuged briefly at 800 x g to remove cells and debris, and added into a 100 mm plate containing untransfected COS-7 cells. The final volume of medium in the plate containing SRUC3 was brought up to 10 ml with cell culture medium. Cells were then incubated at 37°C, and 200 μl aliquots of media were taken at intervals for Renilla luciferase assay.

2.5. Immunoblot analysis of Renilla luciferase

To confirm synthesis of full-length mutant luciferases, immunoblot analysis of secreted Renilla luciferases was performed after fractionation of total COS-7 cell lysates using SDS-PAGE as previously described (Liu et al., 1997). To investigate whether the introduced mutations affected disulfide bond formation, lysates were fractionated under oxidizing conditions in the absence of 2-mercaptoethanol in the loading buffer.
3. Results

3.1. Construction and expression of mutant secreted Renilla luciferase genes in mammalian cells

Four new secreted Renilla luciferases were obtained after site-specific mutagenesis (Fig. 1): SRUC1 (cysteine 52 to alanine), SRUC2 (cysteine 101 to alanine), SRUC3 (cysteine 152 to alanine), and SRUC4 (cysteines 52, 101 and 152 to alanine). Immunoblot analysis of lysates of COS-7 cells expressing the mutant luciferase genes and fractionated under reducing conditions indicated that all mutant proteins appeared to be full-length, and synthesized at similar levels (Fig. 2A). Under oxidizing conditions, immunoblot analysis indicated that substitution from cysteine to alanine in SRUC1, 2, and 3 had an effect on disulfide bond formation, as suggested by the presence of higher molecular weight forms of these luciferases (Fig. 2B, lanes 4, 5, and 6).

3.2. Bioluminescence activity of cell lysates and culture media containing secreted Renilla luciferases

Renilla luciferase activity present in lysates and culture media (serum free medium + 1% FBS) of COS-7 cells synthesizing the SRUC1, 2, 3, 4 and SRUC luciferases was measured 48 h after transfection (Fig. 3). Measurements of Renilla luciferase activity in cell lysates revealed that only two of the four luciferase mutants, SRUC1 and SRUC3, exhibited significant intracellular bioluminescence activities (Fig. 3 A). While SRUC1 exhibited only half the activity of the original SRUC luciferase, SRUC3 exhibited approximately three times the activity of SRUC luciferase. Measurements of Renilla luciferase activity in culture media revealed that medium containing SRUC3 luciferase had a bioluminescence activity up to 100-fold higher than medium containing the SRUC luciferase (Fig. 3 B). Time course measurements of
*Renilla* luciferase activity in culture media confirmed this finding (Fig. 4). Thirty hours after transfection, bioluminescence activity of media containing SRUC luciferase decreased, while bioluminescence activity of media containing SRUC3 luciferase remained at a similar level for at least 50 h.

3.3. Stability of secreted *Renilla* luciferases in culture medium

To determine whether the increased bioluminescence activity of SRUC3 in culture medium was a result of increased stability, the activities of secreted SRUC and SRUC3 luciferases were measured over time at 37°C in serum free medium + 1% FBS. From the data obtained (Fig. 5), the half-lives of SRUC and SRUC3 were calculated to be 14 and 82 h, respectively.

3.4. Cell culture medium dependency of secreted *Renilla* luciferase activity

We had previously observed that the activity of SRUC luciferase was dependent on the type of culture medium in which cells were growing (Liu et al. 1997). To determine whether this was also the case for SRUC3 luciferase, COS-7 cells were transfected with plasmid pND2-SRUC3, grown either in DMEM medium +10% FBS or serum-free medium + 1% FBS for 48 h, and *Renilla* luciferase activity was measured in both cell lysates and culture media. Lysates and culture media of cells grown in DMEM + 10% FBS showed a two fold and a more than five fold reduction in light emission, respectively, when compared to lysates and culture medium of cells grown serum free medium + 1% FBS (Fig. 6).
Discussion

The work presented here provides further evidence that Renilla luciferase is remarkably well-suited to be engineered for secretion by mammalian cells as a functional enzyme. This property is probably the result of its low molecular weight and the small number of cysteine residues it contains. The importance of protein size for the functional secretion of a normally intracellular protein is suggested by the examples of firefly luciferase and the photonprotein apoaequorin. Although firefly luciferase (MW$_r$: 65 kDa) appears to be secreted by mammalian cells when fused to preproinsulin, it does not give a bioluminescence activity in cell culture media which is higher than that resulting from the release of intracellular Renilla luciferase synthesized by the same cells, indicating low activity of the secreted insulin-firefly luciferase fusion protein (Pouli et al., 1998). In contrast, the apoaequorin protein (MW$_r$: 21 kDa) from the jellyfish Aequorea victoria, retains full function even when secreted as part of a fusion with an immunoglobulin heavy chain polypeptide (Casadei et al., 1990).

In addition to protein size, the number of cysteine residues present in an intracellular protein which is to be engineered for secretion should also be an important factor. Disulfide bond formation plays a determining role during folding of secreted proteins (Clarke and Fersht, 1993), and can increase protein stability (Thornton, 1981). Disulfide bond engineering for increased protein stability is an often-used approach, which can be met with more or less success (Wells and Powers, 1986; Matsumura et al., 1989; Van den Burg et al., 1994; Mansfeld et al., 1997). Loss of protein function after introduction of a disulfide bond has been attributed to side effects of the substitution of an amino acid by cysteine per se (Wells and Powers, 1986; Mitchinson and Wells, 1989; Matsumura et al., 1989; Van den Burg, 1994), as well as to conformational constraints imposed by the introduced disulfide bond (Katz and Kossiakov, 1986; Villafranca et al.,
1987). It seems therefore likely that the larger and the more cysteine residues an intracellular protein has, the less probable it will be for that protein to retain its function when secreted.

Wild-type *Renilla* luciferase protein contains three predicted cysteine residues in its amino acid sequence (Lorenz *et al*., 1991). For comparison, apoaequorin, another example of an intracellular protein that was successfully modified for functional secretion, also contains three cysteine residues. When secreted, apoaequorin has several apparent molecular weights in a non-reducing polyacrylamide gel, presumably because of the presence of different disulfide bonds within different conformations (Inouye and Tsuji, 1992). Together with our unpublished observation that secreted *Renilla* luciferase could sometimes form large aggregates in mammalian cells, this result suggested that the odd number of cysteine residues in secreted *Renilla* luciferase could affect its bioluminescence activity. Each cysteine residue in *Renilla* luciferase was therefore replaced individually by alanine, to obtain three different secreted *Renilla* luciferases with a single disulfide bond. In addition, a luciferase with all three cysteines replaced by alanine was also generated.

Immunoblot analysis of lysates of COS-7 cells synthesizing these proteins and fractionated under oxidizing conditions revealed that substitution of the various cysteines by alanine had an effect on disulfide bond formation (Fig. 2B). Surprisingly, SRUC luciferase, presumed to contain three cysteine residues, was detected as a single species (Fig. 2B, lane 3) with the same apparent molecular weight as cytosolic *Renilla* luciferase (lane 2). In contrast, SRUC1, 2, and 3, which are presumed to contain two cysteine residues, were present in addition as higher molecular weight species (lanes 4, 5, and 6, respectively), likely corresponding to multimers of *Renilla* luciferase. We can only speculate that removal of a cysteine residue may have affected the folding of secreted
Renilla luciferase and/or its interaction with folding machinery (e.g., protein disulfide isomerases, molecular chaperones) in a way that prevented efficient folding of the mutant luciferases. Higher molecular weight forms of secreted mutant Renilla luciferases might also be explained by the presence of undetected additional cysteine residue(s) that would be available for intermolecular cross-linking. However this seems less likely in view of the fact that luciferase SRUC4, which contains all three substitutions, did not form similar complexes (lane 7).

Measurements of Renilla luciferase activity from lysates of simian cells expressing the different gene constructs revealed that two out of the four mutant luciferases, SRUC1 and SRUC3, had significant bioluminescence activity (Fig. 3A). In contrast, SRUC2 and SRUC4 showed levels of light emission merely above background. In the absence of a known three dimensional structure of Renilla luciferase, it is difficult to speculate on the reason causing this loss of activity. However, since SRUC2 and SRUC4 luciferases are the only two mutants containing a cysteine to alanine substitution at position 101 (Fig. 1), it is possible that the loss of activity was due to a side effect of this particular mutation.

Between the two functional mutants, SRUC3 was the only luciferase to show a higher bioluminescence activity in cell lysates when compared to the original construct SRUC. When Renilla luciferase activity was measured in culture media of the same cells, this difference in light emission was even more pronounced (Fig. 3 B). Increased bioluminescence activity of media containing the SRUC3 luciferase when compared to media containing the SRUC luciferase could be observed shortly after transfection (Fig. 4). In both cases, light emission reached a plateau approximately 30 h after transfection. However, while light emission from SRUC-containing medium started to
decline after that time, light emission from SRUC3-containing medium remained relatively constant.

Altogether, these data suggested that the increased bioluminescence activity of SRUC3 when compared to SRUC was due mostly to increased stability of the SRUC3 luciferase. Stability measurements of both luciferases confirmed that this was the case (Fig. 5). The half-life of SRUC and SRUC3 luciferases secreted by simian cells at 37°C into serum free medium +1% FBS was calculated to be 14 and 82 h, respectively. We cannot exclude the possibility that part of the light emission measured with media containing SRUC3 resulted from increased specific activity of SRUC3, and /or increased rate of secretion of that protein, as it was shown that removal of cysteine residues from a secreted protein could result in the latter (Omura et al., 1992).

We had previously detected higher light emission from serum-free medium + 1% FBS where SRUC-secreting simian cells were growing than from DMEM medium + 10% FBS containing similar cells (Liu et al., 1997). In addition, aggregation of SRUC in mammalian cells had been observed after one rare instance when no light emission could be detected from transfected cells grown in DMEM medium +10% FBS. Aggregation could be observed only when using non-reducing SDS-PAGE, suggesting that it was the result of intermolecular disulfide bonds (data not shown). We had not seen this occasional loss of activity with cells grown in DMEM + 10% FBS and synthesizing SRUC3 luciferase. However, we did detect a medium-dependency of the SRUC3 luciferase activity (Fig. 6). The decrease in light emission observed when SRUC3 is secreted into DMEM medium +10% FBS when compared to SRUC3 secretion into serum free medium + 1% FBS is not entirely due to a difference in stability of the luciferase in the two different media, as it was shown that stability of wild type *Renilla* luciferase does not vary drastically in these two media (Liu et al., 1997). Preliminary data suggest that it
is the presence of FBS which is responsible for this variation, as removal of FBS from DMEM resulted in increased light emission in the culture medium (data not shown). This effect appears to be specific to secreted Renilla luciferase, because it was not observed with SEAP.

Our results show that removal of a single cysteine residue from the original secreted Renilla luciferase construct (SRUC) resulted in a dramatic increase in stability of the mutated enzyme. This increase in stability could be the result of the formation of a unique disulfide bond within secreted Renilla luciferase. Although possible, it seems unlikely that this increase in stability is a direct effect of the single cysteine to alanine substitution at position 152. This is suggested by the example of a thermolysin-like protease from Bacillus thermoproteolyticus, where increase in stability obtained after the engineering of a single disulfide bond (Mansfeld et al., 1997) was found to be equivalent to the introduction of a total of five amino acid substitutions (Eijsink et al., 1995).

From a practical standpoint, the increased light emission associated with increased stability of SRUC3 makes this luciferase a much improved marker protein. Secreted Renilla luciferase appears to be a good candidate to be used in conjunction with SEAP in a dual assay system of gene expression, where one marker protein is used for studying the activity of a promoter of interest, and the other marker protein is used for normalization between experiments. The “dual-luciferase assay system,” which integrates the intracellular firefly and Renilla luciferases, illustrates the advantages that a dual assay system using light emission-catalyzing proteins has to offer, i.e., simplicity of use and sensitivity (Sherf et al., 1996). A similar dual assay system based on secreted reporter proteins could make this kind of assay even more simple to perform.
Acknowledgment

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References


Figure 1. Secreted *Renilla* luciferases used in this study. The SRUC preprotein (342 aa) is a fusion of the human interleukin-2 leader peptide (IL, aa 1 to aa 23) to wild type *Renilla* luciferase (Liu et al., 1997), which contains three predicted cysteine residues. SRUC1, 2, and 3 are mutants of SRUC where in each case a cysteine residue was replaced by alanine. In the case of SRUC4, all three cysteines were replaced by alanine. N: amino terminus; C: carboxyl terminus.
**Figure 2.** Immunoblot analysis of lysates of COS-7 cells transiently expressing genes encoding secreted *Renilla* luciferases. Total protein from cell lysates were fractionated under reducing (A), or oxidizing (B) conditions using 12% SDS-PAGE, transferred to a membrane, and reacted with rabbit polyclonal antibodies raised against wild-type Renilla luciferase. Cells were transfected with the plasmid vector only (lane 1), PND2-RUC encoding wild-type intracellular Renilla luciferase, (lane 2), PND2-SRUC (lane 3), PND2-SRUC1 (lane 4), PND2-SRUC2 (lane 5), PND2-SRUC3 (lane 6), and PND2-SRUC4 (lane 7); lane 8 contains wild type *Renilla* luciferase isolated from *Escherichia coli* as control.
A

1 2 3 4 5 6 7 8

36 kDa

B

1 2 3 4 5 6 7 8

50 kDa

36 kDa

36 kDa
Figure 3. *Renilla* luciferase activity in mammalian cell lysates (A) and culture media (B). Light emission was measured 48h after transfection, with COS-7 cells synthesizing SRUC (1), SRUC1 (2), SRUC2 (3), SRUC3 (4) and SRUC4 (5). In A, cells were lysed in 1 ml buffer, and 3 µl lysate aliquots were taken for analysis. In B, 200 µl aliquots of cell culture media from the same plates were taken for the assay, with the exception of cells transfected with plasmid PND2-SRUC3, where only 2 µl aliquots of media were taken and diluted into 198 µl of culture medium for measurement of light emission, to keep readings within the scale of the luminometer. The number of relative light units (RLU) obtained in that case was multiplied by 100 before being used as data. In all cases, measurements were normalized using intracellular firefly luciferase activity. Data represent the average of five different experiments.
Figure 4. Time-course measurement of secreted *Renilla* luciferase activity in cell culture medium. Simian COS-7 cells were transfected with plasmid pND2-SRUC1 (■), SRUC2 (♦), SRUC3 (▲), SRUC4 (x), and SRUC (●).
Figure 5. Stability of secreted luciferases in cell culture medium. Already secreted SRUC (▲) and SRUC3 (■) luciferases were incubated at 37°C in culture dishes containing COS-7 cells growing in serum free medium + 1% FBS, and Renilla luciferase activity was measured over time.
Figure 6. Dependence of SRUC3 bioluminescence activity on cell growth condition. COS-7 cells were transiently transfected with plasmid pND2-SRUC3, grown in DMEM medium +10 FBS (DMEM) or serum free medium + 1% FBS (SFM), and Renilla luciferase activity was measured in cell lysates and cell culture media 48 h after transfection.
CHAPTER FIVE

VISUALIZING AND QUANTIFYING PROTEIN SECRETION WITH A RENILLA LUCIFERASE-GFP FUSION PROTEIN

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The format of this chapter follows the journal style of Journal of Luminescence.

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Visualizing and quantifying protein secretion
with a *Renilla* luciferase-GFP fusion protein

(Eukaryotic gene expression; reporter gene; green fluorescent protein; protein secretion)

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Abbreviations: aa: amino acid(s); SEAP: secreted alkaline phosphatase; bp: base pair(s); CMV: cytomegalovirus; FBS: fetal bovine serum; KDa: kilodalton(s); M\(_{r}\): molecular weight; PAGE: polyacrylamide gel electrophoresis; PCR: polymerase chain reaction; SDS: sodium dodecyl sulfate; SRUC: secreted *Renilla reniformis* luciferase.

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Abstract

The luciferase enzyme from *Renilla reniformis* and the green fluorescent protein from *Aequorea victoria* (GFP) have been used to monitor gene expression and protein translocation in a variety of cell types. We have previously shown that an engineered form of *Renilla* luciferase (SRUC) is secreted as a functional enzyme by mammalian cells, and that fusing non-secreted *Renilla* luciferase with GFP yields a chimeric protein retaining light-emission properties similar to that of unfused *Renilla* luciferase and GFP. In the work presented here, the SRUC protein was fused with GFP to determine whether it could be used to both visualize and quantify protein secretion in mammalian cells. Simian COS-7 and Chinese hamster ovary (CHO) cells were transiently transfected with DNA constructs encoding a secreted or an intracellular version of a *Renilla* luciferase-GFP fusion protein. *Renilla* luciferase activity was measured from COS-7 cell lysates and culture media, and GFP activity was detected in CHO cells using fluorescence microscopy. Data indicated that the SRUC-GFP fusion protein was secreted as a chimeric protein that had both *Renilla* luciferase and GFP activity. Secreted *Renilla* luciferase-GFP fusion protein has the potential of being a powerful marker for the study of protein secretion in mammalian cells.
Introduction

Protein secretion by eukaryotic cells is a complex cellular event which is still not fully understood. Several methods and marker proteins have been used to study many aspects of the protein secretion pathway. In recent years, the use of the green fluorescent protein from *Aequorea victoria* (GFP) has permitted direct visualization of protein secretion within a living cell. GFP (Mr: 27 kDa) is a soluble cytosolic protein that fluoresces (lambda max=480) when exposed to U.V. light, and does not require any substrate or cofactor to be functional (Nishiuchi et al., 1999). The only requirement for GFP fluorescence is atmospheric oxygen (Heim et al., 1994), which is necessary for formation of the GFP chromophore. GFP has been fused with several naturally secreted peptides and proteins, including human chromogranin B (Kather and Gerdes, 1995; Wacker et al., 1997; Lang et al., 1997), trans-Golgi network 38 protein (Girotti et al., 1996), vesicular stomatitis virus glycoprotein (Presley et al., 1997), neuropeptide Y (Lang et al., 1997), and insulin (Pouli et al., 1998). Fusion of GFP with these proteins has permitted scientists to follow their translocation within mammalian cells in real time, and has yielded valuable information on the kinetics and mechanisms of protein secretion.

However, it is difficult to quantify the amounts of these secreted protein fusions when using GFP activity due to the poor sensitivity of its assay, combined with background fluorescence in cell culture media. Fusing a secretory signal peptide with a luciferase fused to GFP could allow one to follow visually the fate of the secreted fusion
protein by observing GFP fluorescence within a cell, and at the same time to quantify secretion by measuring luciferase activity in the cell culture medium.

The *Renilla* luciferase enzyme (Mr: 36 kDa) is a monomeric intracellular protein which has been used as a marker of gene expression in cells of various origins (Lorenz *et al.*, 1991; Mayerhofer *et al.*, 1995; Srikantha *et al.*, 1996; Lorenz *et al.*, 1996). This luciferase catalyzes emission of visible light (lambda max= 509 nm) in presence of oxygen and coelenterazine (Matthews *et al.*, 1977). Fusion of a human interleukin-2 (IL-2) signal peptide with *Renilla* luciferase was shown to cause its secretion by mammalian cells as a functional enzyme (Liu *et al.*, 1997). In addition, *Renilla* luciferase-GFP and GFP-*Renilla* luciferase fusion proteins are known to retain both luciferase and GFP activity (Wang *et al.*, 1997). Altogether, these results suggested that *Renilla* luciferase was a good candidate for the construction of a secreted luciferase-GFP fusion.

In this work, we present evidence that an IL-2 signal peptide-*Renilla* luciferase-GFP fusion protein can be secreted by mammalian cells while retaining luciferase and GFP activities, based on measurements of *Renilla* luciferase activity and visualization of GFP fluorescence. These results indicate that secreted *Renilla* luciferase-GFP fusion protein could be used as both a quantitative and qualitative marker of protein trafficking in mammalian cells, and that active *Renilla* luciferase can be secreted as part of a chimeric protein.
Materials and Methods

1. Plasmid construction

Plasmid pND2-RUC/GFP was constructed by ligating a Renilla luciferase-GFP gene fusion as a 1690 bp BamHI-Apal DNA fragment from plasmid pGEM-5zf (+)-RG2 into the BamHI-Apal sites of plasmid PND2-RUC (the constructions of pGEM-5zf (+)-RG2 and pND2-RUC will be described elsewhere), generating plasmid PND2-RUC/GFP (Figure 1A). Plasmid pND2-SRUC/GFP was constructed by ligating a Renilla luciferase-GFP gene fusion as a 1690 bp. BamHI-Apal DNA fragment from plasmid pGEM-5zf (+)-RG2 into the BamHI-Apal sites of plasmid pND2-SRUC (construction pND2-SRUC will be described elsewhere), generating plasmid pND2-SRUC/GFP (Figure 1B).

2. Cell culture and transfection

COS-7 and CHO cells were grown in 60 mm tissue culture plates containing 3 ml DMEM medium with 10 \% Fetal Bovine Serum (FBS). Cells were subcultured 24 hours before transfection and grown until they reached 60\% confluence. The ProFection calcium phosphate system (Promega, Madison WI) was used for transfection, with 30 \( \mu g \) of plasmid DNA per plate. Cells were washed with cold phosphate-buffered saline (PBS) 8 hours after transfection, 3 ml serum free medium (Sigma) + 1\% FBS was added to each plate, which were then incubated at 37\(^{\circ}\)C in a humidified 5\% CO\(_2\) incubator.
3. Immunoblot analysis

COS-7 cells grown in 60 mm plates were washed twice with cold PBS 48 hours after transfection, and harvested by scraping into 100 μl 2x gel-loading buffer (0.1% Bromophenol Blue, 1M sodium phosphate, pH7.4, 4% SDS, 20% glycerol). Cells were then lysed by sonication on ice (2x 10 seconds), samples were placed in boiling water for 3 min, and centrifuged at 10,000 x g at room temperature for 10 min to pellet cell debris. For each sample, 25 μg total protein was loaded on a 12% SDS-polyacrylamide gel for electrophoresis. Fractionated proteins were then transferred by electroblotting onto a nylon membrane, reacted with a monoclonal antibody (mAb) raised against Renilla luciferase, and detected on an x-ray film using chemiluminescence.

4. Renilla luciferase assay and GFP visualization

The Renilla luciferase assay was performed as previously described (Liu et al, 1997). Five microliters total protein of cell lysate and 200 μl of culture medium were used per assay. For GFP visualization, CHO culture medium was changed to FX12 medium 34 hours after transfection, and GFP fluorescent signal was detected using an inverted fluorescent microscope (Zeiss, Oberkochen, Germany).
Results

1. Construction of the SRUC-GFP fusion protein and immunoblot analysis

DNA sequences encoding the interleukin-2 signal peptide (23 aa), *Renilla* luciferase (320 aa), and GFP (242 aa) were ligated to generate a 1.7 Kb DNA fragment carrying the *sruc-gfp* gene, encoding a 583 aa fusion protein. The *sruc-gfp* gene construct was then expressed in simian COS-7 cells together with a *Renilla* luciferase-GFP gene fusion lacking the IL-2 sequence (Wang et al., 1997, named *ruc-gfp* in this article), and immunoblot analysis of total cell lysates was performed using a mAb raised against *Renilla* luciferase protein. Results indicated that cells expressing *ruc-gfp* (Fig.2 lane 2) and *sruc-gfp* (Fig.2 lane 3) gene constructs synthesized proteins with a molecular weight corresponding to that of the RUC-GFP fusion protein, and that cells expressing *sruc-gfp* contained lower amounts of luciferase-gfp fusion protein than cells expressing *ruc-gfp*. Altogether, these data suggested that SRUC-GFP fusion protein was synthesized in full-length, properly processed, and secreted.

2. Renilla luciferase assay

*Renilla* luciferase activity was measured with lysates and culture media of cells transfected with plasmids pND2, pND2-RUC/GFP, and pND2-SRUC/GFP. Luciferase activity measured from lysates of cells expressing the *ruc-gfp* gene was approximately 12 times higher than that of cells expressing the *sruc-gfp* gene (Fig.3 A, 2 and 3, respectively). In contrast, luciferase activity measured from culture media of cells
expressing the \textit{sruc-gfp} gene was about 800 fold higher compared to that of cells expressing the \textit{ruc-gfp} gene (Figure 3 B, 3 and 2, respectively). These results were similar to those obtained with COS-7 cells expressing the \textit{sruc} gene alone (Liu \textit{et al.}, 1997), and a further indication that the SRUC-GFP protein fusion was actively secreted by mammalian cells.

3. \textit{Visualization of intracellular GFP fluorescence}

GFP fluorescence was detected in CHO cells expressing the \textit{ruc-gfp} and \textit{sruc-gfp} gene constructs using fluorescence microscopy. Cells transfected with vector pND-2 only showed low levels of background fluorescence (data not shown), while cells expressing the gene fusion constructs showed strong fluorescent signals with distinct intracellular distribution. Cells expressing the \textit{ruc-gfp} gene displayed a diffuse GFP signal in both the cytosol and the nucleus (Fig. 4 A). In contrast, cells expressing the \textit{sruc-gfp} gene displayed a reticular and punctuate pattern of GFP fluorescence throughout the cell (Fig. 4 B). These patterns are characteristic of wild type GFP and secreted GFP fluorescence, respectively, in mammalian cells (see discussion), and indicated secretion of the SRUC-GFP fusion protein by CHO cells.


Discussion

Reporter genes have been widely used to understand many aspects of gene expression in mammalian cells. Genes encoding bioluminescent marker proteins have been particularly useful, because they provide a very sensitive means to detect transcriptional and translational activity of a specific gene, or to localize the presence of a particular gene product within a single cell or multicellular organism. In most cases, intracellular marker proteins are sufficient for a given purpose. However, there are times when a secreted bioluminescent marker protein becomes a primary choice. In recent years, research focusing on intracellular eukaryotic protein trafficking has made increasing use of bioluminescent proteins as new tools to study protein secretion. The green fluorescent protein from *Aequorea victoria* (GFP) has been the light-emitting marker protein of choice for studying protein secretion. Because GFP only requires atmospheric oxygen and U.V. light to be functional, differential uptake of substrate by various membrane-bound structures containing GFP is not a factor affecting fluorescence. More importantly, this feature allows the investigation of protein translocation within a single living cell, and in real time. In combination with photobleaching, which permits measurement of secretory granule mobility (i.e., rate and trajectory) after fluorescence recovery, these properties of GFP have helped to gather new data on protein secretion which were previously difficult to obtain (Levitan, 1998).

For all the advantages that GFP has to offer as a marker of intracellular protein trafficking, there is still a limitation to its use for studies of protein secretion. Measuring
levels of protein secreted into cell culture media based on GFP fluorescence can be difficult, due to the inherent low sensitivity of its assay. It is possible to detect presence of GFP secreted by mammalian cells after blocking its secretion, releasing the blockage, and measuring a peak of GFP fluorescence when GFP accumulated intracellularly is released into the medium (Wacker et al., 1997). However, little or no GFP activity can be detected at steady state levels (Wacker et al., 1997; Pouli et al., 1998). Nevertheless, some luciferases and other light-catalyzing proteins with higher assay sensitivity than GFP can be used to measure secretion in cell culture media readily, such as Vargula hilgendorfii luciferase (Thompson et al., 1990), secreted Renilla luciferase (Liu et al., 1997), secreted apoaequorin (Inouye and Tsuji, 1992), and secreted alkaline phosphatase (Berger et al., 1988). Thus, fusing a secreted luciferase enzyme with GFP appears to be an attractive way of combining the sensitivity of a luciferase assay for measuring protein secretion in a quantitative manner easily, with the ability to directly localize the same secreted protein using GFP fluorescence in situ.

In this work, we have constructed such a chimeric marker protein by fusing secreted Renilla luciferase with GFP. Secreted Renilla luciferase was a candidate of choice for this purpose, because we were aware that a fusion of intracellular Renilla luciferase with GFP (RUC-GFP) was functional in mammalian cells (Wang et al., 1997). The secreted Renilla-luciferase GFP fusion protein, SRUC-GFP, appeared to be synthesized full-length and properly processed in mammalian cells, as determined by SDS-PAGE and comparison with RUC-GFP (Fig. 2). Two lines of evidence indicated
that the secreted *Renilla* luciferase-GFP fusion protein was secreted, and moreover retained the light-emitting functions of *Renilla* luciferase and GFP. First, significant *Renilla* luciferase activity could be detected in cell culture medium only when the interleukin-2 signal peptide was fused to the *Renilla* luciferase-GFP fusion; in its absence, luciferase activity was merely above background (Fig. 3 B). Second, visualization of intracellular GFP activity in mammalian cells synthesizing RUC-GFP and SRUC-GFP fusion proteins showed that while RUC-GFP was present diffusely throughout the cell, SRUC-GFP had a distinct subcellular localization (Fig. 4). The observed distributions observed in each case corresponded to the abundantly documented patterns of fluorescence seen in mammalian cells with unfused intracellular GFP, and secreted GFP in mammalian cells, respectively. The fusion protein RUC-GFP, with a molecular weight of 64 kDa, could freely diffuse into the nucleus without the need for active transport and a nuclear localization signal (Fig. 4 A). In contrast, SRUC-GFP, which has a similar molecular weight, was found in entirely different sites within the cell (Fig. 4 B). Fluorescence from the presumably processed SRUC-GFP was seen in structures most likely corresponding to the Golgi apparatus and secretory vesicles. Future studies using confocal microscopy should reveal the precise nature of these intracellular organelles.

The secreted *Renilla* luciferase-GFP fusion protein represents a significant advance in the development of marker proteins for the study of protein secretion. Using this protein in living cells, one could alter mammalian protein secretion (eg., using
antisense oligonucleotides to modify expression of a gene suspected to be involved in protein secretion, or inhibitory peptides, Ca\(^{++}\), etc.) and follow changes in protein secretion in qualitatively in situ using GFP fluorescence at the single cell level, as well as quantitatively by measuring Renilla luciferase activity in the culture medium at the population level. Because luciferase and GFP activities would come from the same protein, one would be able to compare directly the data obtained for each assay. This is contrast with the use of secreted luciferase and secreted GFP used as separate markers, where such comparison would be difficult to make as a result of differential rate of gene expression, protein secretion or degradation
Acknowledgments

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References


Figure 1. Partial structures of the plasmids used in this study. Ampicillin resistance gene and bacterial origin of replication are not shown. (A) Plasmid pND2-RUC/GFP carries a cDNA encoding the wild type Renilla luciferase-GFP fusion protein. (B) Plasmid pND2-SRUC-GFP carries the SRUC-GFP gene, which encodes IL2SP fused to the wild type RUC/GFP fusion protein.
Figure 2. Immunoblot analysis of total cell lysates using Renilla luciferase mAb. Simian COS-7 cells were transfected with plasmid pND2-RUC/GFP (lane 2), plasmid pND2-SRUC/GFP (lane 3), and vector pND2 only (lane 1). Lane 4 contains 10 ng isolated Renilla luciferase as control. Totally 25 ug of cell lysates were loaded on each well.
Figure 3. Renilla luciferase activity from RUC-GFP fusion protein in cell lysate (A) and in cell culture (B). Light emission was measured with simian COS-7 cells transfected with: 1. pND2 vector only; 2, pND2-RUC-GFP; 3, pND2-SRUC-GFP. Results obtained from three individual transfections are shown. Relative light units (RLU) are shown as per second, per 5 μg total protein in (A), and as per second, per 200 μl medium in (B).
Figure 4. Directly visualization of protein secretion pathway through GFP fluorescence. CHO cells were transfected with plasmid pND2-RUC-GFP (A), and plasmid pND2-SRUC-GFP (B). Thirty-four hours after transfection, CHO cells were visualized under uv microscope.
CHAPTER SIX

COMPARISON OF RENILLA LUCIFERASE, FIREFLY LUCIFERASE, AND SECRETED ALKALINE PHOSPHATASE AS MARKERS OF GENE EXPRESSION AFTER INTRAMUSCULAR DNA INJECTION IN MICE

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The format of this chapter follows the journal style of Journal of Luminescence.
Comparison of Renilla luciferase, Firefly luciferase, and secreted alkaline phosphatase as markers of gene expression after intramuscular DNA injection in mice

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Abbreviations: aa: amino acid(s); SEAP: secreted alkaline phosphates; bp: base pair(s); CMV: cytomegalovirus; FBS: fetal bovine serum; kDa: kilodalton(s); Mₚ: molecular weight; PAGE: polyacrylamide gel electrophoresis; PCR: polymerase chain reaction; SDS: sodium dodecyl sulfate; SRUC: secreted Renilla reniformis luciferase

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Abstract

Four reporter genes encoding secreted alkaline phosphatase, secreted Renilla luciferase, and Firefly luciferase, were transferred into BALB/c mice after intramuscular “naked” DNA injection. Enzymatic assays were performed on muscle and serum samples. Results showed that Renilla luciferase could be used as a reporter protein in mice. Result from organ culture showed that secreted Renilla luciferase were secreted by muscle cells, although there was no signal of secreted Renilla luciferase can be detected from whole blood or serum. Possible strategies to improve secreted Renilla luciferase as an in vivo marker are discussed.
1. Introduction

Bioluminescent reporter genes have emerged as powerful tools to study gene delivering efficiency and promoter regulation. Light-emission from bioluminescent reaction provides a rapid and sensitive assay to quantitatively monitor gene expression. Among several bioluminescent markers, Firefly luciferase and green fluorescent protein (GFP) are the most commonly used ones. Because none of these markers protein can be applied universally under different conditions in different biological systems, it is necessary to develop new bioluminescent markers. During the past few years, Renilla luciferase has been developed as a new member of luciferase family. Isolated from sea pansy *R. reniformis*, an anthozoan coelenterate living in shallow coastal waters of North America (Matthews *et al.*, 1997), *Renilla* luciferase catalyzes a blue-green light emitting bioluminescent reaction. As a gene expression maker, *Renilla* luciferase has been applied in bacterial (Lorenz *et al.*, 1991), plant (Mayerhofer *et al.*, 1995), yeast (Srikantha *et al.*, 1996), and mammalian cells (Lorenz *et al.*, 1996).

However, most gene expression markers mentioned above are intracellular proteins. Host cells carrying these marker genes have to be destroyed before analysis can be conducted. Cell lysis means disruption of experimental continuity, and makes certain experiments, such as time-course experiments, more unreliable. The requirement of cell lysis becomes more unacceptable when gene activity is monitored in an isolated system, such as animal model or human body in gene therapy trials, because it is not practical to repeatedly remove tissue or organ samples for analysis from an animal or human body.
Unlike intracellular marker proteins, secreted markers allow continuous gene expression monitoring on the same group of cells or tissues without cell lysis since signals for marker proteins could be picked up from cell culture medium (in vitro), or body fluid (in vivo). There are several secreted reporter proteins that have been developed, such as secreted alkaline phosphatase (SEAP) (Berger et al., 1990), secreted Renilla luciferase (SRUC) (Liu et al., 1997), Vargula hilgendorfii luciferase (Thompson et al., 1990), apoaequorin (Inouye and Tsuji, 1992), and GFP from Aequoria victoria (Kaether and Gerdes, 1995; Laukkanen et al., 1996). The application of Vargula hilgendorfii luciferase is limited because its substrate is too expensive (about $2,000/g). Requirement for temporary arrest of protein transport and difficulty to quantitatively monitoring gene expression restricted the use of secreted of GFP. SEAP and SRUC are good candidates as secreted reporter protein to quantitatively monitor gene expression. SEAP has been applied as a regular secreted protein for in vitro studies, but to our knowledge there is no report on SEAP for in vivo studies. This in vivo study was conducted to detect secreted Renilla luciferase (SRUC) in BALB/c mice, but no signal of SRUC could be detected from blood/serum samples. To optimize SRUC as a marker protein, SRUC3 has been developed as a mutant form of SRUC with dramatically improved protein stability (Liu et al., unpublished data). Although it may affect SRUC3 to dynamically reflect the change of gene activity, increased stability should allow secreted protein to accumulate to a certain level to be detected during in vivo studies.
There are many different gene-delivering systems for animal study, and intramuscular (IM) injection of "naked" DNA appears to be a simple and safe way to achieve this purpose. IM injection was first reported to transfer genes into skeletal muscle in vivo by direct injection of plasmid DNA without any delivery vehicle such as a lipid shell (Wollf et al., 1990). Compared to virus vectors, direct gene transfer offers several advantages (Levy et al., 1996; Davis et al., 1993). IM DNA injection does not require dividing cells as the retrovirus do, and should have less possibility to create immunological difficulties because plasmid DNA can be purified to homogeneity without external protein. Despite evidence that the DNA remains in an unintegrated circular form, expression has been shown to persist for up to 19 months (Wolf et al., 1992). The epichromosomal structures also minimize the risk of genomic insertional mutagenesis. Studies showed that in mouse skeletal muscle, plasmid DNA transfer holds better efficiency than that of adenovirus and retrovirus.

IM DNA delivering method has been applied to deliver reporter genes into animal model. Firefly luciferase (Wolff et al., 1990; Levy et al., 1996; Perez et al., 1996; Prigozy et al., 1993), chloramphenicol acetyltransferase (Wolff et al., 1990; Wells D. J., 1993; Prigozy et al., 1993), β-galactosidase (Wolff et al., 1990; Nishi et al., 1996; Prigozy et al., 1993), and human placental alkaline phosphates (Perez et al., 1996) have been reported to be directly transferred through IM injection. IM injection has also been applied in DNA based immunization and DNA vaccination against Influenza A (Montgomery et al., 1993; Yankauckas et al., 1993) and Hepatitis B virus (Mancini et al.,
1995; Michel et al., 1995; Davis et al., 1995; Gramzinski et al., 1998). Recent reports showed that delivering plasmid DNA encoding cytokine (Nitta et al., 1998) through IM injection could prevent type I diabetes in non-obese-diabetic mice.

In the work presented here, plasmid DNA encoding four reporter genes, SEAP, Firefly luciferase, Renilla luciferase, and SRUC3, were delivered into BALB/c mice through IM injection. Enzymatic analysis was conducted on blood and muscle samples. Data showed that the signals of all four proteins were detected from muscle cells within 24 hours after injection and signals could last at least for 20 days. Activity of RUC and SRUC3 in muscle cells were in similar range compared to that of Firefly luciferase and SEAP, and it proved that RUC can be used as in vivo reporter protein in mice. However, SRUC3 could not be detected from blood or serum. Due to its high background, the conclusion of detection of SEAP from serum could not be made. Result from organ culture showed that both SRCU3 and SEAP were secreted by muscle cells. SRUC3 has to be further optimized before being applied as secreted markers for in vivo studies.

2. Materials and Method

2.1. Plasmid construction

pND2-Firefly: plasmid pND2-Firefly luciferase was kindly presented by Dr. Malone from University of Maryland at Baltimore.

pND2-SEAP: plasmid pND2-SEAP was constructed by ligating the SEAP gene as a 1708 bp HindIII-Hpal DNA fragment from plasmid pSEAP2-Control (Clontech Inc.,
Palo Alto, CA) into the HindIII-HpaI sites of plasmid pBluescript II KS(+), and was subcloned as a 1750 bp SalI-NotI DNA fragment into compatible sites of plasmid pND2 to generate plasmid pND2-SEAP.

pND2-RUC: plasmid pND2-RUC was constructed by ligating the Renilla luciferase gene as a 980 bp SalI-SmaI DNA fragment into the SalI-EcoRV site of plasmid pND2, generating the plasmid pND2-RUC.

pND2-SRUC3: the construction of pND2-SRUC3 has been described elsewhere.

2.2. DNA preparation

Plasmid DNA constructions were isolated and purified by standard double-round cesium chloride purification method. E.coli transformed with DNA constructs of interest were grown in 2-liter culture medium overnight supplemented with antibiotics. Cells were pelleted and DNA was extracted through standard alkaline-lysis method. DNA was dissolved in double distilled water and CsCl was added at 1g/1ml ratio. Solution was warmed to 30°C to facilitate the dissolution of salt. Zero point eight ml of ethidium bromide (10mg/ml in water) were added for every 10ml DNA/CsCl salutation. The final density of the solution should be 1.55g/ml, and the concentration of Ethidium bromide should be approximately 740 µg/ml.

DNA/CsCl solution was added in to 15 ml high-speed centrifuge tubes and centrifuged at 75,000 rpm for 16 hours at 20°C in XL-Ultracentrifuge (Beckman). Three
layers were formed in the tube and DNA was contained in the clear, red one in the middle. A 27-gauge needle was used to transfer the clear and red solution into another 15 ml high-speed centrifuge tube, and CsCl was added again and DNA-CsCl solution was spun one more time. DNA band was removed and transferred into a 50ml plastic tube. Aluminum foil was used during the procedure to protect the DNA degradation due to visible light.

1-butanol saturated with water was used to remove the ethidium bromide from DNA solution. Equal volume of 1-butanol was added into DNA solution, mixed well with vertex, and upper layer of solution containing Ethidium bromide was removed. This procedure was repeated at least 10 times until all the pink color disappears from both the aqueous phase and the organic phase.

Dialysis tube from Pierce was used to remove CsCl from DNA solution. DNA was dialysis twice in 2 liters TE buffer for 24 hours at 4°C, precipitated with 20% Ammonium Acetate and 3 volumes of ethanol for 5 hours at -20°C followed by centrifugation at 10,000 x g for 30 minutes at 4°C, and was resuspended in double distilled water at final concentration of 2 µg/µl.
2.3 Intramuscular DNA injection

DNA (200 µg/leg) was injected into mice quadriceps muscle through a 27-gauge needle under general anesthesia (Ketamine, 66 mg/Kg body weight, Phoenix Pharmaceutical, Oxylazine, 7.5 mg/Kg body weight, LLOYD Laboratories, and Acepromazine Maleate, 1.5 mg/Kg body weight, Fermenta). One hundred forty-four 7-week-old BALB/c mice (about 25 gm body weight) purchased from Jaskson Lab were maintained in Loma Linda animal care facility during the experiment. Sixteen mice were used as negative control with injection of pND2 vector only. The rest 128 mice were evenly divided into four groups, and plasmid DNA pND2-Firefly, pND2-SEAP, pND2-RUC, and pND2-SRUC3 were injected into each group. Four mice from each group (two from control group) were killed on day 1, 3, 5, 7, 10, 12, 15 and 20 after injection for enzymatic analysis.

2.4. Sample collection

All blood samples were collected through a cardiovascular puncture, stored at 4ºC degree overnight, centrifuged at 1200 x g for 10 minutes. Serums were harvested and stored at -70ºC. Quadriceps muscles (average 0.122 mg) were stored at -70ºC after being removed from mice.
2.5. Enzymatic analysis

2.5.a. Firefly luciferase assay

Firefly luciferase assay system was purchased from Promega and was performed as prescribed (Firefly luciferase system, Clontech, Palo Alto, CA). Muscle samples were homogenized in 1 ml 1x Cell Culture Lysis Reagent (CSLR) (25 mM Tris-phosphate, pH7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100) with OMNI 2000 homogenizer (Omni International Inc., VA, USA). 50 ul of homogenized tissue and 50 ul of serum samples were mixed separately with 100 µl of room temperature Luciferase Assay Reagent. Samples were vertexes 2 seconds and light production was measured in TURNER TD-20e luminometer.

2.5.b. secreted alkaline phosphatase assay

Great EscAPe SEAP Assay Kit was purchased from Clontech and performed as described. 1 ml 1X lysis buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM MgCl₂, and 0.1% Triton X-100) was used to homogenize muscle samples. Fifty µl homogenized tissue and 50 µl serum samples were used in each assay. Tissue and serum samples were mixed with 75 µl 1 x dilution buffer and incubated at 65°C for 30 minutes. Samples were cooled to room temperature by being placed on ice for 2-3 minutes, and then being equilibrated to room temperature. One hundred µl of assay buffer was added to each sample and incubated for 5 minutes at room temperature. One hundred µl of 1.25 mM chemiluminescent substrate CSPD (CSPD: chemiluminescent =1:19) was added to each
sample and incubates for 30 minutes at room temperature before light production being measured in TURNER TD-20e luminometer.

2.5.c. RUC assay

Muscle samples were homogenized in 1ml RUC assay buffer (0.5 M NaCl, 1mM EDTA, 100mM potassium phosphate, pH 7.4). Fifty microliters of homogenized samples were assayed for bioluminescence in a luminometer (Turner TD-20e) after rapid injection of 500 μl 1 μM coelenterazine (Molecular Probes Inc.). Two hundred microliters aliquots of serum were assayed for bioluminescence as described above.

2.6. Organ culture of mouse quadriceps muscle

Twenty 7-week-old BALB/c mice (about 25 gm body weight) were purchased from Jackson Lab and were maintained in Loma Linda animal care facility. Eight mice were injected with plasmid pND2-SRUC3, six mice were injected with plasmid pND2-RUC, and other six mice were injected with plasmid pND2-SEAP. To each case, 200 μg DNA was injected into each leg of mice. Seven days after injection, mice were killed and quadriceps muscles were removed, cut into 4 pieces, and directly incubated in 1 ml DMEM medium with 10% Fetal Bovine Serum and 1% antibiotic-antimycotic (100x, with 10,000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per ml in 0.9% sodium chloride, Sigma-Aldrich Co. LTD., Irvine, KA) under normal cell culture condition (37°C, 5% CO₂). Two hundred fifty microliters of medium (200 μl for RUC
assay, 50 μl for SEAP assay) were taken at regular time interval and were used immediately for functional assay. Two hundred fifty microliters of fresh medium were added as replacement to each plate. SEAP assay and RUC assay were performed on all samples serving as negative control to each other.

2.7. Whole body imaging of mice after intramuscular injection of luciferase genes

To check if it is possible to directly visualize the gene expression of reporter genes after intramuscularly DNA injection, two 6-week-old mice were injected with Firefly and Renilla luciferase, respectively. Two hundred μg of plasmid DNA were injected into the right leg of the mouse, and 200 μg of DNA of PND2 vector were injected into the left leg as negative control. Ninety six hours after injection, mice were anaesthetized, stabilized on a piece of white paper, and substrate was injected into quadriceps muscles and tail vein for whole body imaging (regular amount was used for firefly luciferase, and 100 μl 50 μM coelenterazine for Renilla luciferase). Gene expression of Firefly luciferase was monitored directly through mice body, but mouse skin had to be pulled back to capture the signal of Renilla luciferase.
3. Results and discussion

There is an increasing demand to develop new gene expression markers in the past few years. Secreted bioluminescent marker proteins provide a rapid, sensitive, and non-invasive assay to monitor gene activity. This becomes more important when experiments are being conducted in animal model systems (Naffakh et al., 1996).

*Renilla* luciferase was engineered to be secreted by mammalian cells, and genetic modification was conducted to improve its protein stability. To find out whether *Renilla* luciferase can be used as an intracellular marker protein in comparison of Firefly luciferase, and whether SRUC3 can be applied as secreted reporter protein in animal studies, plasmid DNA encoding RUC, SRUC3, SEAP, and Firefly luciferase cDNAs were transferred into BALB/c through intramuscular injection. Expression of reporter proteins was monitored by measuring photon from light-emission from muscle and blood/serum samples.

Based on the data from muscle samples, the sensitive range of RUC assay are comparable to that from Firefly luciferase and SEAP. RUC and SRUC3 displayed the same trend of gene expression: the signal of luciferase can be detected 24 hours after injection, peaks at seventh day, and starts to drop after that. Firefly luciferase displayed a rather different expression pattern: signal did not reach peak until twelve days after injection. Signal could still be detected at least for 20 days after injection for all three markers. There is no significant background for both Firefly luciferase and *Renilla* luciferase from muscle samples.
Due to its unusually high background in muscle samples, no expression pattern can be drawn regarding SEAP expression. Only data from day ten and day fifteen could be considered statistically significant compared to background.

Bioluminescent assays were also performed on whole blood and serum. No signal of Firefly luciferase and secreted Renilla luciferase was detected from either whole blood or serum samples. Due to its wide-range high background from control samples, SEAP signals were detected only in 7 out of 32 serum samples, and there is no gene expression pattern could be drawn from these data. Because SEAP is supposed to be heat reliable at 65°C and resistant to Homoaragine, these two treatments have been used to eliminate the background during in vitro studies. The high background from muscles and serum samples may come from endogenous alkaline phosphate or unknown protein(s), which is (are) capable of interacting with substrate for SEAP. For an unknown reason, heat and Homoaragine were not able to eliminate the background caused by unidentified protein(s). This unexpected high background could limit the application of SEAP as an in vivo marker.

It is not clear why the secreted Renilla luciferase was not detected from blood or serum, but there are several reasons will be discussed. Muscle cells may produce but not secrete Renilla luciferase protein. The secretion efficiency may have been too low and most of the proteins could be retained inside of the muscle cells. Renilla luciferase may have been secreted into blood, but the gene expression level is not high enough to reach the level of detection. Dilution of secreted protein by body fluid of mice may also reduce
the chance of detection of Renilla luciferase. It is known that there are size and charge requirements in kidney for proteins to be cleared out, Renilla luciferase may have been cleared out through kidney after being secreted into blood because of its relative low molecular weight. Protein may have been degraded by proteases in the blood or liver. It may have been targeted to certain organ(s), or may not be stable enough in blood to accumulate to a threshold level for being detected. Overall, several or none of these reasons could explain the undetectable Renilla luciferase activity in the blood or serum.

To explore whether SRUC3 and SEAP protein were secreted by muscle cells or not, in vitro organ culture experiments of isolated muscle tissues were conducted. Gene expression and protein secretion were monitored up to 72 hours. Even though the activity level of SRUC3 and SEAP in medium were fairly low compared to that from in vitro studies, data from Renilla luciferase and SEAP assay on organ culture medium showed that both SRUC3 and SEAP were secreted by muscle cells (Figure 3). The background for SEAP from culture medium is noticeably lower than that from blood and muscle samples. It is not clear why there is high in vivo background for SEAP, but it will limit the application of SEAP as in vivo marker protein.

Whole body imaging experiments showed that Renilla luciferase could be used with Firefly luciferase in a dual-marker system for in vivo study. Because of the relative larger size of substrate for Renilla luciferase and subsequently poor penetration into muscle cells, the signal of Renilla luciferase was weaker than that from Firefly luciferase.
Consistent with the result from Renilla assay on blood samples, there was no signal for secreted Renilla luciferase in mice.

If its sensitivity level can be improved, SRUC3 would be a better candidate as in vivo marker protein compared to SEAP since it has no background in the mouse. The following procedures can be conducted to improve Renilla luciferase assay system:

(1) Application of more efficient vector system with stronger promoter would increase the odds for detection of secreted Renilla luciferase. Systemically delivering system, such as T-cell-based-protein-secretion system, may also provide a better chance to detect SRUC3.

(2) More studies on Renilla luciferase protein structure may reveal new direction to improve protein stability, sensitivity, specific activity and secretion efficiency. Modification could be conducted to change the codon usage in Renilla luciferase and optimize it for mammalian cell expression. More knowledge about protein structure itself may lead to find a better-match substrate with increase activity. It was reported that in vitro secretion rate of SEAP was as high as 98%. Based on data obtained from in vitro and in vivo studies, the secretion rate of Renilla luciferase was much lower than that. Modification of SRUC3 needs to be done to achieve a higher secretion rate.

(3) Renilla luciferase fusion protein with increased molecular weight may elude possible host clearance through kidney. Circulating proteins with lower molecular weight may be cleared from kidney, although the exact cutoff size is not clear. Since albumin (60 kDa) is retained in kidney, fusion of Renilla luciferase with another gene to
generate a fusion protein with molecular weight higher than 60 kDa may prevent the clearance of SRUC3. (4) The conduction of a more sensitive luminometer will also increase the chance of picking up signals from blood/serum samples.

In summary, Renilla luciferase can be applied for in vivo studies as a sensitive bioluminescent marker. We are currently modifying secreted Renilla luciferase and optimizing detecting system for SRUC3 to be used as a secreted marker in animal models.
ACKNOWLEDGEMENTS

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Figure 1. Partial structure of the plasmids used in this study. Ampicillin resistance gene and bacterial origin of replication are not shown. (A) Plasmid pND2-\textit{lac} carries a cDNA encoding the wild type Firefly luciferase protein. (B) Plasmid pND2-\textit{ruc} carries a cDNA encoding the wild type \textit{Renilla} luciferase protein. (C) Plasmid pND2-\textit{seap} carries a cDNA encoding the secreted alkaline phosphatase. (D) Plasmid pND2-\textit{sruc3} carries a cDNA encoding the mutant form of secreted \textit{Renilla} luciferase. CMV, cytomegalovirus promoter; SEAP, secreted alkaline phosphatase; RUC, \textit{Renilla} luciferase; SRUC3, mutant form of secreted \textit{Renilla} luciferase.
A. pND2-\textit{luc}

\begin{center}
\begin{tikzpicture}
    \node[rectangle, draw] (cmv) at (0,0) {CMV};
    \node[rectangle, draw, right of=cmv, xshift=20mm] (firefly) {Firefly luciferase};
    \draw[->] (cmv) -- (firefly);
\end{tikzpicture}
\end{center}

B. pND2-\textit{ruc}

\begin{center}
\begin{tikzpicture}
    \node[rectangle, draw] (cmv) at (0,0) {CMV};
    \node[rectangle, draw, right of=cmv, xshift=20mm] (renilla) {\textit{Renilla} luciferase};
    \draw[->] (cmv) -- (renilla);
\end{tikzpicture}
\end{center}

C. pND2-\textit{seap}

\begin{center}
\begin{tikzpicture}
    \node[rectangle, draw] (cmv) at (0,0) {CMV};
    \node[rectangle, draw, right of=cmv, xshift=20mm] (seap) {Secreted alkaline phosphatase};
    \draw[->] (cmv) -- (seap);
\end{tikzpicture}
\end{center}

D. pND2-\textit{sruc3}

\begin{center}
\begin{tikzpicture}
    \node[rectangle, draw] (cmv) at (0,0) {CMV};
    \node[rectangle, draw, right of=cmv, xshift=20mm] (sruc3) {Secreted \textit{Renilla} luciferase};
    \draw[->] (cmv) -- (sruc3);
\end{tikzpicture}
\end{center}
Figure 2. Time-course of reporter gene activity appearance in muscle cells after DNA injection. Plasmid DNA encoding RUC (A), SRUC3 (B), Firefly luciferase (C), and SEAP (D), were injected into 5-week-old BALB/c mice. Muscle cells were isolated at regular time interval, homogenized in assay buffer, and light emission was monitored by luminometer. Relative light units (RLU) are shown as per second, per 50 ul homogenized samples.
Figure 3. Organ culture of muscle cells carrying plasmid DNA encoding SEAP, RUC, and SRUC3. Seven days after injection, quadriceps muscle were isolated from BALB/c mice, and incubated in DMEM + 10% FBS. Culture media were taken at 24, 48, and 72 hours after incubation, and SEAP assay (A) and RUC assay (B) were performed on all samples. Medium from muscle carrying DNA encoding SEAP serve as negative control in RUC assay, and medium from muscle carrying DNA encoding RUC/SRUC3 serve as control in SEAP assay. Relative light units (RLU) are shown as per second, per 200 μl cell culture medium.
Figure 4. Whole body imaging of BALB/c mice intramuscularly injected with Firefly and *Renilla* luciferase DNA. Five days after injection, mice were stabilized, and substrate (100 μl of room temperature Luciferase Assay Reagent for Firefly luciferase, and 100 μl 50 μM coelenterazine for *Renilla* luciferase) were directly injected into quadriceps of mice. Imaging was taken immediately in NightOwl Image system.
CHAPTER SEVEN

INTRAMUSCULAR INJECTION OF PLASMID DNA ENCODING INTRACELLULAR OR SECRETED GLUTAMIC ACID DECARBOXYLASE CAUSES DECREASED INSULITIS IN THE NON-OBESE DIABETIC MOUSE

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⁴ Professor in the Immunology Center, Department of Pathology, School of Medicine, Loma Linda University who directed the research, and isolated pancreatic and spleen tissue from NOD mice for immunoassay and insulitis scoring experiments

⁵ Student’s mentor and chairman of research committee who directed research and supervised the preparation of manuscript and dissertation

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Intramuscular injection of plasmid DNA encoding intracellular or secreted glutamic acid decarboxylase causes decreased insulitis in the non-obese diabetic mouse

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**Summary**

Our goal is to determine whether gene vaccination can be used for the treatment of insulin-dependent diabetes mellitus (IDDM), an autoimmune disease. In this work, weanling non-obese diabetic (NOD) mice, an animal model system for the study of IDDM, received intramuscular injections of naked plasmid DNA encoding either intracellular or secreted human glutamic acid decarboxylase (GAD), one of the major autoantigens recognized during the onset of IDDM. Seven weeks later, each pancreas was scored for insulitis, an inflammation indicative of the disease. Mice treated with either type of gad gene-carrying plasmid showed a significant decrease in the severity of insulitis when compared to controls. These results suggest that vaccination using autoantigen-encoding genes may provide a means of treating IDDM.
**Introduction**

Insulin dependent diabetes mellitus (IDDM), or type I diabetes, is a disease with high morbidity and mortality that affects 1 in 300 persons in North America, with a prevalence ever increasing in small children (for a review see Mandrup-Poulsen, 1998). Although also called juvenile diabetes because it often affects young people, a similar disease has been diagnosed in patients 50 years of age and older (Molbak et al., 1994). IDDM is thought to be caused by both genetic and environmental factors, and is associated with the autoimmune destruction of insulin-producing beta cells found in the pancreatic islets of Langerhans. Loss of these insulin-secreting cells results in the inability to metabolize glucose, leading to hyperglycemia and ketoacidosis, which in turn cause a variety of debilitating and life-threatening ailments such as blindness, kidney disease, heart attack, stroke, and neuropathy. Although injection of the hormone insulin can prolong life of IDDM patients, it does not provide a cure for the disease, likely due to lack of proper regulation of insulin levels within the body. A cure for IDDM could be achieved if the destruction of beta cells were averted.

IDDM has been characterized as an autoimmune disease based on the observations that patients suffering from this illness have high levels of islet cell autoantibodies in their sera (Bottazo et al., 1974), and chronic mononuclear cell infiltration of their pancreatic islets (Gepts and Lecompte, 1981). Presence of autoantibodies can be detected years before the onset of symptoms, and is considered to
be diagnostic for IDDM (Maclaren, 1988), although it does not always imply occurrence of the disease. In humans, the nature of these antibodies varies with age: autoantibodies against insulin and tyrosine phosphatase IA-2 are associated with early childhood, glutamic acid decarboxylase (GAD) and islet cell cytoplasmic protein autoantibodies with late childhood and adolescence, while late onset can be associated with other typical immune markers (Mandrup-Poulsen, 1998). Inflammatory infiltration of the islets (insulitis) and beta cell destruction are due mostly to T lymphocytes, both CD4\(^+\) helper and CD8\(^-\) cytotoxic (Itoh et al., 1993; Peakman et al., 1994), and result in loss of islet cell mass. When this cell mass drops below 10% of normal, hyperglycemia and ketosis develop.

A large part of what is known about IDDM comes from studies of animal model systems, in particular the non-obese diabetic (NOD) mouse. The NOD mouse possesses most of the characteristics of human IDDM, such as genetic predisposition due to MHC II linkage, development of insulitis with infiltration of T lymphocytes selectively toxic to insulin-producing beta cells, and humoral reactivity to beta cells (for a review see Bowman et al., 1994). However, unlike humans, NOD mice have a strong gender bias in the appearance of the disease: 91% of females NOD/Lt mice manifest diabetes at 250 days of age, while only 21% of males show a similar symptom at that age (Baxter et al., 1991).
Studies of NOD mice (Kaufman et al., 1993; Tisch et al., 1993) and patients (Baekkeskov et al., 1990) indicate that the GAD protein is a major autoantigen recognized during the onset of IDDM. GAD is an enzyme found mostly in neurons (Erlander et al., 1991) and pancreatic islet cells (Christgau et al., 1991), where it catalyzes the synthesis of gamma-aminobutyric acid (GABA). GABA is an inhibitory neurotransmitter in the central nervous system, and may be a paracrine signaling molecule in the pancreas. Two forms of GAD are encoded by different genes in mammals: a 65 kDa (previously called 64) and a 67 kDa (previously called 65) molecular weight form. GAD65 is a membrane-anchored intracellular protein, while GAD67 is found soluble in the cytosol (Christgau et al., 1991; Christgau et al., 1992). Both GAD65 and GAD67 are recognized by the immune system of IDDM patients (Baekkeskov et al., 1990; Honeyman et al., 1993). In addition, the first T cell response against beta cell antigens in 4-week old NOD mice is against GAD65 (Kaufman et al., 1993; Tisch et al., 1993), and both CD8+ cytotoxic (Panina-Bordignon et al., 1995) and CD4+ T helper1 (Th1) (Tabata et al., 1998) lymphocytes specific for GAD65 can be found in patients suffering from IDDM. Together with the finding that adoptive transfer of GAD-reactive T cells can cause diabetes in NOD/SCID mice (Zekzer et al., 1998), these observations strongly indicate that GAD65 plays an important role as an autoantigen during onset of IDDM.
The NOD mouse serves not only as a model to study IDDM, it is also an excellent system for the development of new methods for preventive therapy of this form of diabetes. Such therapies include immunosuppression, immunostimulation, tolerance induction, manipulation of hormonal/dietary milieu, and anti-inflammatory agents (Bowman et al., 1994). In this work, we have investigated whether gene vaccination could be used to prevent insulitis in the NOD mouse. Specifically, we have used intramuscular injection of “naked” plasmid DNA encoding human GAD65 and SGAD55, an engineered secreted form of this protein. We report that injection of DNA encoding these proteins resulted in significant decreases in insulitis, suggesting the possibility that this form of gene therapy might be useful to prevent clinical manifestation of IDDM.

Results

Construction of a secreted form of human GAD65

Extracellular antigens can be used for tolerization or for suppression of MHC class II restricted Th1 inflammatory response, probably through a MHC class II restricted CD4+ Th2 lymphocyte response, as Th1 and Th2 responses appear to be mutually exclusive (Mosmann and Sad, 1996). Therefore, two genes encoding GAD proteins that had the potential of being secreted by mammalian cells were constructed.

The first construct consisted of the leader peptide from human interleukin-2 (IL-2) protein fused to full-length human GAD65, generating a fusion protein encoded by the
sgad65 gene. This leader sequence was previously shown to cause secretion by mammalian cells of normally intracellular proteins (Okano et al., 1990; Liu et al., 1997). However, because GAD65 is a membrane-anchored protein, the protein region responsible for the anchoring could have interfered with secretion. The sequence corresponding to approximately the first 100 amino acids (aa) of human GAD65 contains a Golgi-targeting sequence (Solimena et al., 1994), as well as cysteine residues that are palmitoylated and responsible for membrane anchoring (Christgau et al., 1992). In addition, this sequence is not recognized by autoantibodies from IDDM patients (Richter et al., 1993). The first 88 aa of the human GAD65 protein were therefore deleted, and the remainder of the protein was fused to the IL-2 leader sequence, generating a fusion protein (SGAD55) encoded by the sgad55 gene (Fig. 1 B).

Simian COS-7 cells were transiently transfected with the two gene constructs coding for these proteins, and immunoblot analysis of intracellular GAD protein was performed using a monoclonal human GAD65 antibody. Results confirmed the synthesis of SGAD65 (Fig. 2 A, lane 3), and of the lower molecular weight SGAD55 (Fig. 2 A, lane 4). To determine whether SGAD65 and SGAD55 were secreted by mammalian cells, proteins from COS-7 cells transiently transfected with the different gene constructs were labeled in vivo with 35S-methionine. Culture media from these cells were then used for immunoprecipitation using the same antibody used for immunoblot analysis, and immunoprecipitates were fractionated using sodium dodecyl sulfate polyacrylamide gel
electrophoresis (SDS-PAGE). Results showed that SGAD55 protein was immunoprecipitated from cell culture media (Fig. 2 B, lane 3). In contrast, no secreted SGAD65 protein could be detected (Fig. 2 B, lane 4). The gene construct sgad55 was therefore selected for further use in animal experiments.

Effects of intramuscular injection of GAD-encoding genes on insulitis and cytokine profile

Each of four groups of three-week old female NOD mice received injections of one of four plasmid DNAs, and injections were repeated after three days. Mice were injected with either plasmid vector only, plasmid vector carrying gene sruc3 encoding a stable mutant (JL and AE, unpublished data) of a secreted soft coral luciferase (Liu et al., 1997), the human gad65 gene, or the sgad55 gene encoding secreted GAD protein. The sruc3 gene was used as control for possible non-specific effects of synthesis of a plasmid-encoded antigen on insulitis. Another group of NOD mice was used as a non-injected control (N.B. this group was kept in a different animal room at Loma Linda University, and at a different time).

Mice were killed when 10 weeks old for histopathological analysis, insulitis scoring, and immune assay. None of the mice had developed diabetes, as determined by urine and blood glucose analysis (data not shown). Figure 3 shows representative islets illustrating the levels of insulitis that were observed. Figure 4A shows that a significant
reduction in the severity of insulitis was detected in mice receiving injections of plasmids carrying the gad65 and sgad55 genes, when compared to the three control groups. In addition, mice injected with these gad genes also had a higher percentage of insulitis-free islets (Fig. 4B). Cytokine profiles of GAD65-stimulated spleen lymphocytes (splenocytes) tended to support the histological findings. While the Th1-type cytokines (IFNα and IL-2) were not different between groups (Fig. 5 A and B), IL-4 production (Th2-type) was higher in the gene-vaccinated groups (Fig. 5 C, 3 and 4) than in the controls (Fig. 5 C, 1 and 2), when challenged in vitro with recombinant human GAD65 protein.

Discussion

Gene vaccination consists of the introduction and expression of a gene into an organism, with the purpose of generating an immune response against its encoded product. The simplest way of achieving this purpose is to use the method of intramuscular or subcutaneous “naked” DNA injection, originally presented as a means of expressing plasmid-encoded genes after direct injection of DNA into mouse muscle (Wolff et al., 1990). This method has since been used to generate immune responses to a wide variety of antigens, such as human immunodeficiency virus 1 glycoproteins, and malarial circumsporozoite protein (for a review see Tighe et al., 1998). Although the majority of studies have focused on infectious diseases, “naked” DNA gene vaccination
can also be applied to studies of alloimmunity (Geissler et al., 1994) and treatment of cancer (Condon et al., 1996). Recently, gene vaccination was used to suppress the symptoms of autoimmune encephalomyelitis in rats through synthesis of an autoantigenic peptide (Lobell et al., 1998).

Glutamic acid decarboxylase (GAD) is thought to be a major autoantigen contributing to the onset of insulin-dependent diabetes mellitus (IDDM), and injection of GAD protein can delay the onset of the disease in NOD mice (Kaufman et al., 1993; Tisch et al., 1993; Elliot et al., 1994; Petersen et al., 1994; Sai et al., 1996). In addition, similar results are obtained with oral feeding of the protein (Ma et al., 1997; Ramiya et al., 1997). In this work, we investigated whether expression of genes encoding two forms of human GAD could cause reduction of insulitis in the NOD mouse, an inflammation of pancreatic islets which is characteristic of IDDM in this model.

Two gad genes were used for expression in muscle tissue: a gad65 cDNA encoding human GAD65 protein, and sgad55, a gene construct based on gad65, encoding the interleukin-2 (IL-2) leader sequence fused to a GAD65 protein lacking its first 88 amino acids (SGAD55) (Fig. 1). The N-terminal region of GAD65 was removed in the SGAD55 fusion protein because we suspected that it could interfere with its secretion, since this region contains a Golgi-targeting and membrane-anchoring sequence. This was confirmed by the finding that a fusion of the IL-2 leader sequence to full-length GAD65 could not be detected in the culture media of mammalian cells expressing its encoding
gene (Figure 2B, lane 4). In contrast, SGAD55 protein was detected in culture media of cells expressing the sgad55 gene (Fig. 2B, lane 3).

Intracellular and secreted forms of GAD65 were used in this study because of the known differences in the type of immune response that intra- and extra-cellular antigens can generate: intracellular antigens are presented by MHC class I molecules and generate a CD8+ cytotoxic T lymphocyte response, while extracellular antigens are presented by MHC class II molecules on the surface of antigen presenting cells, generating a CD4+ helper T lymphocyte response (Tighe et al., 1998). Although secreted proteins are synthesized within a cell, they appear to be less likely to be presented by MHC class I molecules than cytosolic proteins (Yewdell et al., 1998).

Synthesis of intracellular GAD65 by muscle cells was not expected to affect insulitis. Considering the small number of muscle cells able to express injected genes, the levels of intracellular GAD65 protein found in injected and non-injected mice NOD should not have differed greatly enough to generate an immune response influencing T cell infiltration of islets. This supposition was corroborated by the finding that in NOD mice transgenic for murine gad65, only those mice showing the highest levels of transgene expression could exacerbate insulitis and diabetes (Geng et al., 1998). In contrast, secretion of SGAD55 could have caused either reduced insulitis (through an anti-inflammatory Th2 response) or increased insulitis (through an inflammatory Th1
response), depending on the levels of extracellular antigens attained (Hosken et al., 1995).

Our results show that injections of gad65-carrying plasmids caused a reduction of insulitis similar, if not greater, to that resulting from injections of sgad55-carrying plasmid (Fig. 4). A variety of non-exclusive mechanisms are thought to lead to Th1 and Th2 immune responses after plasmid DNA injection, such as release of antigens from intact cells expressing the plasmid-carried gene, or from lysed cells after a cytotoxic T lymphocyte response, and direct transfection of antigen presenting cells (Davis et al., 1993; Xiang et al., 1994; Condon et al., 1996; Gregoriadis et al., 1997). Our results suggest that one (or both) of the latter two putative mechanisms was likely to be responsible for the similar reduction of insulitis after intramuscular injection of gad65 or sgad55 gene, since neither the intra- nor extra-cellular nature of the plasmid-encoded GAD antigens appeared to affect the extent of insulitis differently.

To determine the nature of the immune response generated in plasmid-treated NOD mice, cytokine secretion by splenocyte was measured after challenge with recombinant human GAD65 protein. Splenocytes of all plasmid-injected mice secreted similar levels of Th1-specific IFNα (Fig. 5A) and IL-2 (Fig. 5B). However, cells from mice receiving injections of gad65 or sgad55 genes maintained higher levels of Th2-specific IL-4 than the controls when challenged with GAD65 autoantigen (Fig. 5C). No differences in splenic memory cell numbers (immunophenotyping) or blood levels of
cytokines could be demonstrated between groups (data not shown). These results suggest that the reduced levels of insulitis observed after injection of gad65 or sgad55 genes could have been the result of a Th2-mediated response. This would be in accordance with the observation that suppression of insulitis is associated with elevated synthesis of IL-4 and IL-10 (for a review see Rabinovitch, 1998), and that suppression of the diabetogenic response in NOD mice after injection of GAD65 protein is mediated by the induction of GAD65-specific regulatory Th 2 cells (Tisch et al., 1998). Failure to detect systemic changes in circulating cytokine levels (IFNα 75-1365 pg/mL) and splenic phenotypes (13-15% CD62Lneg CD44pos T helper cells) was expected in light of the well-established organ-specificity of this disease. Further work on the cellular infiltrate should reveal more relevant information.

Immunomodulatory gene therapy has been previously considered as a possible approach for the prevention of IDDM. In one study, islet-specific Th1 cells transduced with engineered retroviruses carrying a gene encoding the anti-inflammatory cytokine IL-10 was able to cause reduced insulitis and delayed onset of diabetes when injected into NOD mice (Moritani et al., 1996). In contrast, intramuscular injection of plasmid DNA encoding IL-10 did not cause reduced insulitis, but did result in delay of diabetes onset (Nitta et al., 1998). In another study, intramuscular injection of DNA encoding TGF-β1 caused both reduced insulitis and delayed onset of diabetes (Piccirillo et al., 1998). Our data suggest that intramuscular injection of DNA coding for an IDDM autoantigen could
also be used for this purpose. Plasmid injection offers potentially both therapeutic and economical advantages. Injection of plasmid DNA could permit the development of plasmid “cocktails” encoding combinations of different autoantigens and immunomodulating cytokines. When compared to injection of isolated proteins, the availability, quality, and cost of these therapeutic proteins would not be a concern, since their synthesis would occur within the host. Clearly, injection of plasmid DNA is a promising approach for suppressing symptoms of IDDM or other autoimmune diseases in the future.

Materials and Methods

Gene and plasmid construction

The sgad65 gene encodes a fusion of the leader peptide from human IL-2 to full-length human GAD65 protein. This gene was constructed by ligating an 89 base pair (bp) DNA fragment encoding the first 23 amino acids of IL-2 (isolated previously by PCR from human cell line A293 as described by Liu et al., 1997) in frame with a 1.8 Kilobase pair (Kb) NcoI-XhoI DNA fragment carrying a human GAD65 cDNA. The sgad55 gene encodes a fusion of the leader peptide from human IL-2 to a truncated version of human GAD65 with 88 aa deleted at its N-terminus. Two oligonucleotides were used to amplify the 89 bp DNA fragment encoding the IL-2 leader peptide from gene sgad65, IL-01 (TTT TCT AGA ATG TAC AGG ATGCAA CTC CTG) and IL-03 (TTT ACG CGT
AAG TAG GTG CAC TGT TTG TGA). IL-03 introduced a MluI site which was used to clone the PCR product in frame with the MluI-XhoI 1.5 Kb DNA fragment encoding GAD55, the truncated version of human GAD65. The identity of PCR products and gene fusion junctions were confirmed using automated DNA sequencing.

For cell culture work, the gad65, sgad65, and sgad55 genes were cloned under transcriptional control of the cytomegalovirus (CMV) promoter into plasmid vector pLNCX (Miller and Rosman, 1989). For muscle injection, all genes were cloned under transcriptional control of the CMV promoter in plasmid PND2, a vector known to provide high gene expression in muscle tissues (Gary Rhodes and Robert Malone, unpublished data).

Mammalian cell culture and transfection

Simian COS-7 cells were grown in 60 mm tissue culture dishes containing 3 ml DMEM medium with 10% fetal bovine serum (FBS). Media were changed 3 hrs prior to transfection when cells were 70% confluent. Cell transfection was performed using the ProFection calcium phosphate system (Promega, Madison, WI) using 40 ug of plasmid DNA per plate. Cells were incubated with the DNA-calcium phosphate complex for 6 hours, washed twice with phosphate-buffered saline (PBS), and 3 ml DMEM medium +10% FBS was added into each plate. Culture plates were then incubated for 48 hours before harvesting cells and media for analysis.
**Immunoblot analysis**

Simian COS-7 cells were washed twice with cold PBS 48 hrs after transfection, and harvested in 100 μl hot 2x gel-loading buffer (100mM Tric.HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.01% bromophenol blue) using a rubber policeman. Cells were lysed by sonication, boiled for 3 min, and lysates were centrifuged at 1000 x g for 10 min to pellet cell debris. Twenty microliters from each sample was loaded on a 12% SDS-polyacrylamide gel for fractionation. Proteins were then transferred onto a nylon membrane by electroblotting, and a GAD65 monoclonal antibody was used to detected GAD protein using a method previously described (Liu et al., 1997).

**Protein radiolabeling and immunoprecipitation**

To detect secreted GAD proteins in cell culture media, $^{35}$S-methionine (specific activity>1000 Ci/mmol, from DuPont NEN, Boston, MA) was used to label total cell protein from COS-7 cells. Media were removed after incubation with the DNA-calcium phosphate complex, and cells were rinsed twice with 1 x PBS and once with medium without methionine and serum. Cells were then incubated in 3 mL DMEM medium without methionine + 1% dialyzed FBS for 20 min to deplete intracellular pools of endogenous methionine. One hundred microcuries of $^{35}$S-methionine was then added directly into media for protein labeling. Cells were incubated for 24 hours before being
harvested. Media were collected and concentrated using a Centricon spin column (15 kDa molecular weight cut-off, from Amicon) to a final volume of 500 ul. $^{35}$S-labeled GAD protein was immunoprecipitated from media using the Protein A Immunoprecipitation Kit (Boehringer Mannheim, Indianapolis, IN) and a monoclonal antibody raised against human GAD65. Immunoprecipitates were fractionated in a 12% SDS-polyacrylamide gel, and protein bands were detected by exposure to X-ray film.

**Isolation of plasmid DNA for muscle injection**

Plasmid DNA was amplified in *Escherichia coli* strain DH5α, using the alkaline-lysis method, and isolated by standard double-round cesium chloride purification (Maniatis *et al.*, 1989). The quality and quantity of DNA was determined by UV spectrophotometer ($A_{260}/A_{280}$ ratio greater than 1.8) and by agarose gel electrophoresis. Plasmid DNA was dissolved under sterile conditions in double distilled water at a final concentration of 2 µg/ul, and stored at -20°C.

**Intramuscular DNA injection**

Three-week old female NOD mice were purchased from Taconic Laboratories (Germantown, NY) and kept at Loma Linda University animal facilities. Mice were injected with DNA (200 µg/100 µl/leg) into each quadriceps muscle with a 27-gauge
needle under general anaesthesia (Ketamine, 66 mg/Kg body weight, from Phoenix Scientific, S' Joseph, MO; Oxylazine, 7.5 mg/Kg body weight, from LLOYD Laboratories, Shenandoa, IO; and Acepromazine Maleate, 1.5 mg/Kg body weight, from Fermenta Animal Health Co., MO), and injections were repeated three days later. Urine glucose levels were monitored weekly with Clinistix Reagent Strips for Urinalysis (Bayer Corporation, Elkhart, IN). Mice were killed for insulitis scoring at the age of 10 weeks, and blood glucose levels were checked with ACC-CHEK Advantage (Boehringer Mannheim Corporation, Indianapolis, IN).

Histopathological analysis of insulitis

Pancreatic tissues were fixed with 10% buffered Formalin, stained with hematoxylin, and counterstained with eosin, and an average of fifteen islets/mouse were scored. A 7-level semi-quantitative scoring scale (Zhang et al., 1991) was used for insulitis scoring: 0, normal islet tissue without any detectable T cell infiltration; 1, focal peri-islet T cell infiltration with less than one-third of the peri-islet area; 2, more extensive peri-islet T cell infiltration with less than two-thirds of the peri-islet area; 3 peri-islet T cell infiltration with more than two-thirds of the peri-islet area; 4, intra-islet T cell infiltration with less than one-third of the islet area; 5, intra-islet T cell infiltration with less than two-thirds of the islet area; 6, severe intra-islet T cell infiltration with more than two-thirds of the islet area. Scoring of 1-3 indicated peri-insulitis, and scoring of
4-6 indicated intra-insulitis. Scoring was conducted using the double-blind method by two different scorer.

*In vitro challenge of splenocytes*

Lymphocytes were flushed from splenic pulp and washed in complete media (RPMI, 10% FBS, P/S, 2% L-Glutamine, and 4 x 10^(-5) M 2-mercaptoethanol). In a 24-well plate, 1x10^6 cells in 1 mL complete media (unstimulated control) or 1 mL GAD65 (1.5 ug/ml) were cultured (37°C, 5% CO_2) for 72 hrs. Cell culture supernatants and blood plasma (from terminal bleeds) were assayed by standard sandwich ELISA (Endogen, Woburn, MA) for IFNα, IL-2 and IL-4.

*Immunophenotyping of splenocytes*

Since antigen-specific memory cells of the Th1-type T lymphocytes express CD44 and lose expression of CD62 ligand (CD62L) (Mocci and Coffman, 1997; Bradley *et al.*, 1992), splenocytes were stained with three fluorochrome-conjugated monoclonal antibodies (Becton Dickinson, Immunocytochemistry Systems, San Jose, CA) to CD4, CD44 and CD62L. After red cells lysis, the phenotypes were analyzed by 3-color flow cytometry. Phenotyping controls included untreated (autofluorescence) and isotype antibody-treated cells (nonspecific staining). CD4 T cells (phycoerythrin) were back-gated and these were analyzed for the expression of CD44 (PerCP) and CD62L (FITC).
Statistical analysis

Comparison between groups was done using a ONE-WAY ANOVA and Duncans post-hoc test for multiple comparisons.
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**Figure 1.** Partial gene constructs used for intramuscular DNA injection. Three genes were placed under transcriptional control of the cytomegalovirus promoter (CMV) into an expression plasmid: *gad65*, encoding a wild type intracellular human GAD65 protein (A), *sgad55*, encoding a fusion of the IL-2 leader peptide (IL2-LP) to a truncated human GAD65 protein (B), and *sruc3*, encoding a secreted *Renilla* luciferase (C).
Figure 2. Detection of GAD proteins from lysates (A) and culture media (B) of mammalian cells grown in vitro. A. Immunoblot analysis of simian COS-7 cells transiently transfected with different gad genes. Cells were transfected with plasmid vector only (lane 1), plasmid carrying genes gad65 (lane 2), sgad65 (lane 3), or sgad55 (lane 4). Lane 5 contains a truncated version of GAD65 isolated from Escherichia coli as control. Total cells lysates were fractioned using SDS-PAGE, transferred onto a membrane, and reacted with a mouse monoclonal antibody raised against wild-type human GAD65, and subsequently to a secondary antibody bound to alkaline phosphatase for chemiluminescent detection. B. Immunoprecipitation of 35S-Methionine-labeled proteins from culture media. Culture media from COS-7 cells transiently transfected with plasmid vector only (lane 1), plasmid carrying gene gad65 (lane 2), sgad55 (lane 3), or sgad65 (lane 4) were used for immunoprecipitation using the same antibody used in A. Immunoprecipitates were then fractionated using SDS-PAGE and exposed to X-ray film.
Figure 3. Histopathological examination of pancreatic islets. Islets with score 0 (A), and 3 (B) from a GAD65-treated mouse are shown for comparison with islets with score 5 (C) and 6 (D) from a control animal. Arrows point to T cell infiltration.
Figure 4. Insulitis scores of 10-week old female NOD mice. Severity of insulitis is presented based on a 0-7 scoring scale (A), and as percentage of islets showing intra-, peri-, or no insulitis (B). Insulitis was scored with untreated mice (group 1, n=5), mice receiving injections of plasmid vector only (group 2, n=5), or vector carrying gene sruc3 (group 3, n=6), gene gad65 (group 4, n=7), or gene sgad55 (group 5, n=7). Data are presented as the mean score ± SEM. When individually compared to control groups 1 (*), 2 (**), or 3 (** *), group 4 or 5 showed statistically significant differences (P value <0.05). No statistically significant differences were found among groups 1, 2, 3, or groups 4 and 5.
A

Reduction of insulitis

![Graph showing reduction of insulitis scores with error bars and statistical significance markers.](image-url)
Figure 5. Cytokine profile of GAD65 protein-stimulated splenocytes. Splenocytes from mice receiving injections with plasmid vector only (1), or plasmid carrying the sruc3 (2), gad65 (3), or sgad55 (4) genes were stimulated with 1.5 ug/ml of isolated recombinant human GAD65 protein. After 72 hours, culture supernates were assayed for IFNγ (A), IL-2 (B), and IL-4 (C).
CHAPTER EIGHT

CONCLUSION

In this work, proteins have been engineered for secretion to develop a non-destructive marker for monitoring gene activity in a biological system both \textit{in vitro} and \textit{in vivo}, and to investigate whether DNA vaccination with plasmid DNA encoding a secreted protein can be used to treat an autoimmune disease.

The development of reporter genes, especially those encoding light-catalyzing proteins involved in chemiluminescence (CL) and bioluminescence (BL), has greatly enhanced our ability to monitor the transcriptional activity of cloned promoter sequences after their introduction into target cells or organs. Although the activity and amount of reporter gene products are an indirect measurement of the transcriptional properties of the test DNA, it is generally considered that reporter gene activity is directly proportional to transcriptional activity (Alam and Cook, 1990).

Chemiluminescent marker proteins such as \(\beta\)-galactosidase were among the first reporter proteins to be utilized to monitor gene expression (Edlund \textit{et al.}, 1985). However, bioluminescent reporter proteins have become the most commonly used ones in recent years. Bioluminescent marker proteins are enzymes (luciferase) catalyzing the light emission reaction, or light-emitting photoproteins (Kricka, 1991). The overall quantum yield of a chemiluminescent reaction (the number of photons emitted vs number...
of molecules reacting) is generally in the range 1-10%, whereas for bioluminescent reactions the yields are higher, typically in the range 10-30% (Kricka, 1991). Due to this reason, bioluminescent reactions are considered more sensitive than chemiluminescent reactions (Kricka, 1991).

Compared to traditional reporter proteins, bioluminescent reporter proteins provide a more sensitive, rapid, and simple assay for analyzing regulation of gene expression. However, the most commonly used bioluminescent reporter proteins are intracellular proteins, and can only allow for a single assay with one sample. Secreted bioluminescent reporter proteins could overcome this problem and permit continuous assay on the same sample over a period of time without cell disruption. Several secreted reporter proteins have been developed for this purpose, but each one has its own shortcoming. For example, secreted alkaline phosphatase (SEAP) has high in vivo background, the secreted GFP assay has low sensitivity, and the price for substrate for naturally secreted Vargula luciferase is too high (about $2,000/mg vs $90/mg for Renilla luciferase substrate as sold by Prolume Inc., Pittsburgh, PA). Because no reporter protein can be universally applied to all conditions, it would be useful to develop a new secreted bioluminescent reporter protein. Firefly luciferase, the most commonly used reporter protein, is not functional after being fused with signal peptide from insulin for secretion (Pouli et al., 1998). In addition, bacterial luciferase can not be secreted either (Alan Escher, personal communication). Renilla luciferase, an intracellular enzyme catalyzing
a light-emitting reaction in the sea pansy *Renilla reniformis*, has been previously used as a gene expression marker (Lorenz *et al.*, 1991; Srikantha *et al.*, 1996; Lorenz *et al.*, 1996), and was selected in this project to be engineered for secretion.

A functional secreted *Renilla* luciferase (SRUC) was engineered after fusing with human IL-2 leader sequence encoding a signal peptide (Figure 1., Chapter Three). Data from the *Renilla* luciferase assays showed that luciferase activity could only be detected from the culture media of cells transfected with gene encoding SRUC, but not from the cells transfected with gene encoding wild type *Renilla* luciferase. No morphology change or increased cell death was observed on cultured cells after transfection, so it was unlikely that the luciferase activity detected in culture medium was from cell lysis. The immunoblot analysis following immunoprecipitation on culture medium directly revealed the presence of secreted *Renilla* luciferase.

After generating a secreted form of *Renilla* luciferase, we went on to engineer another secreted gene expression marker. This marker was a secreted *Renilla* luciferase-GFP (SRUC-GFP) fusion protein (Figure 1., Chapter Four). It has been shown that *Renilla* luciferase and GFP could work as a dual marker system (Wang *et al.*, 1997). The secreted *Renilla* luciferase-GFP fusion provides a unique way to monitor gene regulation: GFP fluorescent signal can be used to visualize the *in vivo* protein trafficking, and *Renilla* luciferase assay can be used to quantify this process. The SRUC-GFP also indicated that *Renilla* luciferase not only can be secreted as a single protein, it can also be secreted as
part of a chimeric protein. This finding is important for future use of SRUC as an *in vivo* marker of extracellular protein translocation: SRUC could be fused to a protein of interest to identify organs targeted by this protein within an animal.

During the development of secreted *Renilla* luciferase, the secreted luciferase activity could not be detected from culture medium under undefined cell growth conditions. Immunoblot analysis revealed that protein aggregation of secreted protein caused the absence of secreted luciferase in the medium. This phenomenon led to the hypothesis that the odd number of cysteine residues (there are three of them in native *Renilla* luciferase) could have facilitated the formation of intermolecular disulfide bond, and may have affected protein stability and rate of protein secretion. Using site-directed mutagenesis to replace cysteine with alanine, four mutated SRUC genes were generated (Figure 1, Chapter Five). Mutant SRUC3 showed dramatically increased protein signal from both cell lysates and cell culture medium. Compared to the original SRUC, the sensitivity of *Renilla* luciferase assay of SRUC3 in culture medium was increased up to 100 fold after 48 hours. Data showed that SRUC3 was more stable than the original SRUC in culture medium (84 hours half-life for SURC3 compared to 14 hours for SRUC). However, improved protein stability itself could not account for the 100-fold increase of protein assay. Other changed properties, such as increased protein secretion rate, or increased protein enzymatic activity, may also have contributed to the increase in light emission.
Compared to another commonly used secreted marker, SEAP, the sensitivity of the SRUC assay is much lower (Liu et al., 1998). However, the Renilla luciferase assay is much less time-consuming, and is sensitive enough to be used in many experiments (Inouye and Shimonura, 1997).

Because our goal is to develop a secreted bioluminescent marker for animal studies, we transferred genes encoding wild type Renilla luciferase and SRUC3 into BALB/c mice using intramuscular plasmid injection to determine whether Renilla luciferase could be used as an in situ marker. Two other commonly used reporter genes, encoding firefly luciferase and SEAP, were also used in this experiment for comparison with Renilla luciferase. Data showed that both intracellular and secreted Renilla luciferase signals were easily detected in muscles tissues within 24 hours after injection, and the sensitivity of Renilla luciferase assay was within the range of that from firefly luciferase (intracellular protein) and SEAP (secreted protein). Unlike SEAP, Renilla luciferase assay showed low in vivo background. However, a secreted Renilla luciferase signal could not be detected either in whole blood or serum from mice. Data from in vitro whole muscle tissues culture experiment showed that SRUC3 was expressed and secreted by muscle cells at very low levels (Figure 4., Chapter Six). Therefore, more modifications need to be made to further improve SRUC3 before it can be used as a secreted gene expression marker in animals.
The secreted *Renilla* luciferase assay could be improved in several ways. Improving gene expression and/or protein production levels in muscle cells or other possible target organs should increase the sensitivity of the *Renilla* assay. It was reported that gene expression level of GFP could be increased by changing codon usage (Zolotukhin *et al.*, 1996), and we are currently conducting a similar approach to increase the gene expression levels of *Renilla* luciferase. Beside intramuscular DNA injection, other gene transfer techniques, such as cell encapsulation, may be an alternative way to deliver *sruc3* gene into animal model with higher levels of protein secretion. Encapsulated cells expressing the *sruc3* gene may provide higher gene expression and protein production levels for a longer period of time, since cells encapsulated with an immunoisolation shell may evade host immune system and avoid the destruction of cells carrying the gene of interest (Josephs *et al.*, 1999).

It was shown that replacing one amino acid (cysteine to alanine at amino acid No.152) could dramatically improve the stability of *Renilla* luciferase protein in cell lysate and in cell culture medium (Chapter Five). If knowledge of the 3-D protein structure of *Renilla* luciferase were available, we could further modify *Renilla* luciferase to improve its enzymatic activity or protein secretion rate, and therefore increase the sensitivity of the *Renilla* assay. Finding the optimal substrate for *Renilla* luciferase is also important. It was reported that as many as 23 different coelenterazine analogues can be used as substrate for the *Renilla* luciferase assay. The *e*-coelenterazine and *u*-
coelenterazine are the most efficient substrate for *Renilla* luciferase (Inouye and Shimonura, 1997), but highly unstable (Molecular Probe Inc., personal communication). Developing more sensitive luminometer would be another means of improving the secreted *Renilla* luciferase assay. In summary, a promising gene expression marker, secreted *Renilla* luciferase, was developed in this work. With further modification, it may be applied for future *in vivo* studies.

Another aspect of this project was to determine whether DNA vaccination using plasmid DNA encoding an autoantigen could be used to treat an autoimmune disease. Insulin-dependent diabetes mellitus (IDDM) was selected as a disease model in this work. IDDM is caused by the progressive autoimmune destruction of insulin-producing pancreatic β cells (Rabinovitch, 1994). Although the precise mechanism of development of type I diabetes in human and NOD mice remains unclear, it is believed that the presentation of β cell-specific antigens by macrophages and/or dendritic cells to CD4+ T helper cells is the first step in the development of autoimmune IDDM. The activated macrophages secrete IL-12, which stimulates the Th1 cell subset. The activation of Th1 cells will induce cell-mediated immune response against pancreatic cells, and at the same time downregulate the Th2 cell subset. Activated Th1 cells secrete IL-2 and IFN-γ, which activate other resting macrophages to release cytokine and free radicals that are toxic to β cells. During this process, IL-2 and other cytokines induce the migration of CD8+ peripheral T cells to the inflamed islets by inducing the expression of a specific
receptor. The infiltration of T helper cells, macrophages, and CTL into pancreatic islets finally causes insulitis. The combination of macrophages, CD4+ T cells, and CD8+ T cells destroys pancreatic β cells synergistically (Yoon et al., 1997).

Despite progresses made during the last decade, IDDM still remains a disease without a cure. Diabetic patients need daily insulin injection to stay alive. The implantation of pancreatic islet cells was thought to be able to cure type I diabetes, but limited donor availability, host immune attack, and high cost make it difficult for it to be widely applied to diabetic patients (Lacy, 1995; Efrat, 1998). Another potentially attractive approach for patients who have already developed diabetes is β-cell regeneration from islet cell precursors. However, this approach depends on the identification of factors that induce β-cell neogenesis and replication as well as on the development of ways to prevent recurring autoimmunity against the newly formed islets, and is not considered to be clinically applicable any time soon (Efrat, 1998). Delivery of recombinant insulin from encapsulated pancreatic islet should result in higher cell survival rate and longer insulin production/secretion level (Peterson et al., 1998). However, this approach is time-consuming and fairly expensive at this moment (Josephs et al., 1999). Oral tolerance, defined as the delivery of specific autoantigens orally to induce host immune tolerance, has been used to treat type I diabetes in animal model (Hutchings and Cooke, 1998). The mechanisms of oral tolerance are not completely understood. It appears that several outcomes can occur depending on the amount of
antigen administered: with high-dose antigen treatment, T cells clonal deletion or anergy occurs; with low-dose antigen treatment, active suppression happens (Seroogy and Fathman, 1998). Since it is difficult to control gene expression levels after transferring genes encoding antigens of interest in vivo, and therefore hard to control the amount of antigen presented to host immune system, oral immune tolerance does not seem to be the best approach for the treatment of autoimmune disease.

The best strategy to treat IDDM is to prevent the development of disease before its onset. Recently, gene therapy strategies have been applied to the treatment of autoimmune diseases, and organ-specific type I diabetes is a good disease model for gene therapy approaches (Mathisen and Touhy, 1998). Because IDDM is a T-cell-mediated autoimmune reaction, and the Th1/Th2 cell balance seems to be crucial during the development of diabetes, most gene therapy approaches focus on activating Th2 cell and suppressing Th1 cells to reduce T-cell mediated immune attack on pancreatic islet cells (Rabinovitch, 1994). Most of those approaches can be grouped into two categories: cytokine gene therapy, and DNA vaccination (Efrat, 1998; Seroogy and Fathman, 1998; Mathisen and Touhy, 1998).

Since cytokines are crucial during the development of IDDM, it is rational to think that overexpression of anti-inflammatory cytokine should have protective effects on IDDM. Genes encoding several cytokines have been delivered into animal model for diabetes through somatic gene delivery or transgenic approach. However, the results
were variable. Expression of anti-inflammatory cytokines such as TGF-β1 and IL-4, have been shown to prevent IDDM in NOD mice (Piccirillo et al., 1998; Ridgway et al., 1994), and expression of IL-4, IL-10, IFN-β, and TGF-β were able to inhibit experimental allergic encephalomyelitis, another autoimmune disease (Croxford, et al., 1998). On the other hand, the transgene expression of IL-10, an anti-inflammatory cytokine, resulted in an accelerated onset of clinical diabetes accompanies by severe pancreatic inflammation in NOD mice (Lee et al., 1994; Wogensen et al., 1994). One major drawback of cytokine gene therapy is that the production of cytokine is not antigen-specific, and with the current understanding of the cytokine network, the appropriate levels of expression for optimal efficacy are unknown and are difficult to be tightly regulated (Croxford et al., 1998). Thus, cytokine therapy approach may not be the best answer for IDDM.

The ideal approach to treat IDDM should be one that can provoke antigen-specific host immune response and either induce immune tolerance or suppress autoimmune attack on specific organs. Several autoantigens, such as insulin and GAD65, are the ideal targets to generate specific immune response. Purified GAD65 protein has been injected into NOD mice and showed protective effect on insulitis and diabetes (Tisch et al., 1993; Kaufman et al., 1993). It is believed that active suppression after protein injection leaded to this protective effect (Tisch et al., 1998). Transgenic expression of mouse proinsulin II also prevented diabetes in NOD mice (French et al., 1996). However, a transgenic approach is not applicable for clinical trial, and protein injection therapy of
autoantigen has many drawbacks, such as contamination of endotoxin, limited availability, and high cost. DNA vaccination would be an ideal approach to deliver autoantigen into a host to provoke immune responses.

DNA vaccination is defined as direct administration of plasmid DNA encoding antigen(s) specific to a particular pathogen into vaccine recipient (Cohen et al., 1998). Compared with other vaccination methods, DNA vaccination can provoke host specific immune responses with defined synthesis of antigen(s) and without virus or endotoxin contamination. Both humoral and cell-mediated immune responses can be generated with DNA vaccination, and it has been applied to treat a wide variety of genetic and infectious diseases during recent years (Cohen et al., 1998). Recently, DNA vaccination was applied to the treatment of an autoimmune disease, experimental autoimmune encephalomyelitis (Youssef et al., 1997).

In this project, the use of DNA vaccination for preventing IDDM in NOD mice was investigated. Plasmid DNA encoding a major autoimmune antigen, GAD65, was directly injected into three-week-old NOD mice to induce Th2 cell subset mediated humoral response to actively suppress the development of diabetes. However, it was suspected that vaccination with plasmid DNA encoding the membrane-anchored GAD65 protein might not able to achieve this goal because DNA vaccination results in endogenous antigen production, presumably through MHC Class I pathway, and activation of CD8+ CTL cells (Condon et al., 1996). In contrast, secreted antigen might
have been more likely to be taken by antigen-presenting cells (APC), processed and presented through MHC Class II pathway to induce a host humoral response. A plasmid encoding a secreted form of GAD antigen was therefore also constructed. DNA sequence encoding human IL-2 signal peptide was fused to the 5’ end of GAD65 gene to facilitate the secretion of GAD65 protein. However, this fusion gene did not encode a secreted GAD65 protein, probably due to protein modification on the N-terminal region of GAD protein (Christgau et al., 1992). Because there are no major epitopes found at the N-terminal region of GAD65, and because the N-terminal region is responsible for protein anchoring on cell membrane, the DNA sequence encoding the first 88 amino acid of GAD65 protein was deleted and then fused with the signal sequence encoding the IL-2 signal peptide, to generate secreted GAD55 (sgad55). Immunoprecipitation experiments showed that both GAD65 and GAD55 proteins were detected with the predicated molecular weights after transient gene expression, but that only sgad55 encoded a secreted form of GAD protein (Figure 5, Chapter Seven).

Plasmid DNA encoding secreted GAD antigen was then directly injected into quadriceps muscles of three-week-old NOD mice to activate Th2 cells and actively suppress Th1 cell subset. DNA encoding wild type GAD65 protein was injected into another group of mice to compare the vaccination effect from intracellular or secreted antigen. A reporter gene, sruc3, was also injected into NOD mice as negative control for any biological effect induced by GAD proteins.
Seven weeks after injection, mice were killed for insulitis score. Insulitis is a major step in the progression of diabetes, and is defined as islet infiltration by lymphocytes and macrophages (Andre et al., 1996). In the work presented here, pancreatic tissues were isolated from NOD mice, and stained for histopathology analysis. At least 15 islets from each mouse were scored under a double-blind manner, and the raw data were within normal distribution (personal communication with Dr. Grenith Zimmerman, a professor of Biostatistics, Loma Linda University). Insulitis score showed that injection of plasmid DNA encoding human GAD65 and SGAD55 could dramatically reduced the development of insulitis in NOD mice, a specific effect since no such protective effect was observed from control mice injected with sruc3. Several immunoassays were conducted including cytokine assay and in vitro mitogen stimulation. While there was no difference between control mice and GAD-treated mice in IFNγ and IL-2 levels when measured from either blood or in vitro culture, higher levels of Th2-specific IL-4 from spleen cells from mice treated with gad65 or sgad55 genes suggested that the protective effect could result from the induction of CD4+ Th2 cells, which promote humoral immunity and inhibit Th1 T-cell-mediated immunity.

Surprisingly, no meaningful statistical difference of insulitis score between mice treated with GAD65 and SGAD55. A similar phenomenon was previously reported: in vivo transfection of muscle cells with two plasmids encoding either secreted or intracellular form of a malaria antigen generated comparable levels of antibody response
(Haddad *et al.*, 1997). Two hypotheses have been offered to explain this phenomenon. One is that humoral responses may be provoked by release of antigens as a result of cell lysis. Another possibility is that the actual process of genetic immunization directly transfects professional APC, such as dendritic cells (Condon *et al.*, 1996). At this moment, there is no strong evidence supporting either of them.

It was reported recently that injection of plasmid DNA encoding rat GAD65 protein generated a strong humoral immune response in NOD mice, but the incidence of diabetes in NOD mice were not reduced (Wiest-Ladenburger *et al.*, 1998). Compared to the experiment presented here, there are several differences in the protocol used by Wiest-Ladenburger's group which might explain our different results. Their group used small amounts of DNA (100 μg/mice compared to 400 μg/mice in this research work), rat GAD65 instead of human GAD65, and DNA injection was performed at the age of six weeks old instead of three weeks. Since 6-week-old NOD mice have already started to develop insulitis, it may be too late to start the treatment at this age and may lead to no reduction effect on diabetes after DNA immunization with rat GAD65 (Andre *et al.*, 1998).

Overall, the experimental data presented here demonstrated that DNA vaccination could be used in the treatment of an autoimmune disease. However, more work needs to be done to further reveal the molecular and immunology mechanism behind it and to improve this approach.
Future work should focus on two points: to determine if DNA vaccination with plasmid DNA encoding autoantigen GAD has protective effect on diabetes in NOD mice, and if there is a protective effect, to reveal the mechanism of it.

We have shown that DNA vaccination on three-week-old NOD mice can reduce the development of insulitis. To fully evaluate the effect of DNA vaccination on diabetes, we are monitoring the development of diabetes in NOD after the intramuscular injection of plasmid DNA encoding GAD65 and SGAD55 in cyclophosphamide (CYP)-induced diabetes. CYP is widely used clinically as an immunosuppressive agent, particularly in autoimmune disease (Charlton et al., 1989). However, it is found that administration of CYP actually accelerates the development of diabetes in NOD mice. CYP has been used to compress the normal time course of diabetes in NOD mice (Piccirillo et al., 1998; Charlton et al., 1989).

However, even insulitis does not start to develop until 3-4 week of age, it is believed that the autoimmune attack on pancreatic islets starts much earlier than that (Andre et al., 1996). Although we have shown that plasmid mediated GAD treatment could reduce the overall insulitis score, we may still improve this treatment by injecting younger or even newborn NOD mice. We are planning to breed NOD in the Immunology Research Center at Loma Linda University, and we will start DNA injection treatment as early as in the first week after mice is born. The result will be compared to that from intramuscular DNA injection on 3-week-old mice.
Despite of the fact that immunology has made tremendous progresses in recent years, current attempts to optimize naked-DNA vaccination are proceeding without a clear understanding of the mechanism of this form of immunization. One important question is: what kind of cells are transfected after intramuscular DNA injection? Although muscle cells are the main target for plasmid DNA injection, it is possible that APC cells, such as dendritic cells and macrophage, may take in DNA and present antigen directly to host immune system (Condon et al., 1996). One way to determine what type of cells take in and express injected DNA is to use promoter that only expresses in certain type of cells, such as muscle-specific promoter (Miller et al., 1993; Huard et al., 1999; Krempler and Brenig, 1999). Another possible method to answer this question is to inject plasmid DNA encoding GFP marker protein. After encountering antigens, dendritic cells will migrate to thymus or regional lymph nodes where they exhibit potent APC function (Condon et al., 1996). GFP fluorescent signal will allow monitoring of dendritic cells migration after intramuscular DNA injection.

In summary, DNA vaccination is an attractive approach for the induction of immunity against viral infection, tumor, and for the treatment of autoimmune disease. It offers several advantages over other gene transfer method. Among these is the potential for rapid and inexpensive production of large-scale DNA preparation. Such vaccine preparations can be prepared with relative purity and would be significantly more stable than current protein-based vaccines. Furthermore, the use of “naked” DNA vaccines
would be inherently safer than the use of viral mediated gene transfer. However, the molecular mechanism behind DNA vaccination need to be further investigated, and experimental data from studies in NOD mice must be carefully analysed for their applicability to therapeutic intervention in human.
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