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METABOLIC ENGINEERING WITH RECOMBINANT ADENOVIRUSES

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ABSTRACT

Fuel homeostasis in mammals is accomplished by the interplay between tissues and organs with distinct metabolic roles. These regulatory mechanisms are disrupted in obesity and diabetes, leading to a renewed emphasis on discovery of molecular and pharmacologic methods for reversing metabolic disorders. In this chapter, we review the use of recombinant adenoviral vectors as tools for delivering metabolic regulatory genes to cells in culture and to tissues of intact animals. Included are studies on the use of these vectors for gaining insights into the biochemical mechanisms that regulate glucose-stimulated insulin secretion from pancreatic islet β-cells. We also highlight their use for understanding the function of newly discovered genes that regulate glycogen metabolism in liver and other tissues, and for evaluating "candidate" genes such as glucose-6-phosphatase, which may contribute to development of metabolic dysfunction in pancreatic islets and liver. Finally, we discuss the use of adenoviral and related vectors for causing chronic increases in the levels of circulating hormones. These examples serve to highlight the power of viral gene transfer vectors as tools for understanding metabolic regulatory mechanisms.

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INTRODUCTION

Mammals have a remarkable capacity to regulate the levels of circulating metabolic fuels, a phenomenon referred to as fuel homeostasis. This regulation is achieved by interplay between tissues and organs with distinct metabolic roles. Thus, the liver produces glucose in the fasted state, thereby preventing hypoglycemia. In turn, extra-hepatic tissues such as muscle and adipose tissue use glucose in the fed state, preventing hyperglycemia. Adipocytes serve as a reservoir for free fatty acids and, in the fasted state, deliver them via the circulation to the liver, where they are oxidized. Fuel homeostasis is also regulated by interplay between different fuels. For example, fatty acid oxidation in liver produces acetyl coenzyme (Co) A, which is an allosteric inhibitor of pyruvate dehydrogenase and activator of pyruvate carboxylase, leading to activation of gluconeogenesis. Orchestrating this complex array of regulatory events are the pancreatic hormones insulin and glucagon, whose secretion is in turn controlled by metabolic coupling factors within the β- and α-cells of the islets of Langerhans.

Unfortunately, these elegant regulatory mechanisms are profoundly disrupted in obesity and diabetes. The recent surge in the United States in the incidence of obesity, and of its close partner non-insulin-dependent diabetes mellitus (NIDDM), has brought about a renewed focus on metabolic regulatory mechanisms and the need to understand them. It has also encouraged development of molecular and pharmaceutical methods that may reverse disrupted fuel homeostasis. In this chapter, we focus on adenovirus-mediated gene transfer in metabolic research.

Metabolic Engineering of Mammalian Cells—What Can be Learned?

There are many methods for transferring genes into mammalian cells. Recombinant adenovirus is a valuable tool for metabolic engineering because it mediates high-efficiency transfer of genes into mammalian cells, including primary cells with low replicative activity. This allows rapid and efficient modulation of the activity of specific proteins within metabolic pathways, such that

genes are used much like pharmacologic reagents. The following applications of adenovirus-mediated gene transfer are highlighted in this chapter: investigating basic metabolic regulatory mechanisms, understanding the function of newly discovered genes and testing of disease "candidate genes," and delivery of peptide hormones.

Much remains to be learned about how metabolic regulation in specific cells and tissues contributes to fuel homeostasis in whole animals. For example, it is well known that glucose stimulates insulin secretion from β-cells in the islets of the pancreas, and the amount of insulin secreted is proportional to the glycolytic rate. However, the secretion/coupling factors that link glucose metabolism and insulin secretion in β-cells are incompletely understood. Similarly, glucose disposal in liver occurs via the seemingly well-understood metabolic pathways of glycolysis and glycogen synthesis, but recent studies have demonstrated that compartmentation of relevant enzymes and formation and disassembly of complexes of proteins play central roles in regulation of both pathways. Finally, the mechanism(s) by which insulin regulates glucose and lipid metabolism in muscle and adipose tissue has been studied for decades, but detailed understanding will require further testing and refinement of models. Specific examples of the application of recombinant adenovirus technology to each of these areas is provided in this chapter, with particular attention payed to regulatory events in liver and pancreatic islets. Before providing these examples, a brief review of viral gene transfer methods is in order.

Gene Transfer Methods—Why Adenovirus?

GENERAL OVERVIEW OF GENE TRANSFER METHODS There are several methods for introducing foreign DNA into mammalian cells. For studies of metabolic regulatory mechanisms, the efficiency of gene transfer is of major importance. Measurement of metabolic flux with radioisotopic tracers or by nuclear magnetic resonance provides an index of the average activity in populations of cells. Thus, when assessing the metabolic impact of a particular gene product, it is essential to deliver that gene to a majority of cells within the population. Another important consideration is the type of cell that one is trying to transfect. In studies of metabolic regulation, it is desirable to deliver genes to primary cell types that are relevant to control of fuel homeostasis, such as the islets of Langerhans or liver cells. Cells with low replicative activity (e.g. isolated islets of Langerhans) are refractory to stable transfection methods, including murine leukemia virus retrovirus-mediated gene transfer, because of the lack of cell growth. Other nonviral transfection methods such as Ca_2PO_4 coprecipitation, electroporation, and lipofection generally provide gene transfer efficiencies ranging from 2–50% of cells in a population, which is not sufficient for studies of metabolic regulation.

COMPARISON OF ADENOVIRUS TO OTHER VIRAL VECTORS Most virally assisted gene transfer has been carried out with retroviruses (3). These vectors are capable of reasonably efficient gene transfer into a wide variety of cell types, and they integrate into the genome of the host cell, providing the possibility of long-term expression. Several packaging systems for propagation of recombinant murine leukemia virus have been developed, but they have limitations. For metabolic engineering, the foremost limitation is that cell division is required to achieve retrovirus integration into the genome and transgene expression. Furthermore, the efficiency of gene transfer of retroviral vectors is limited, even in dividing cells, where transduction frequencies of approximately 30% are encountered. These features make murine leukemia virus–based retroviral vectors entirely unsuitable for metabolic studies on primary cells in culture with low replicative activity, such as the islets of Langerhans. In addition, this class of retroviral vectors is not suitable for systemic delivery of genes to tissues of intact animals, in part because it has been difficult to produce viral stocks of sufficient titer.

An exciting new vector system is based on lentiviruses, an alternate class of retroviruses that includes HIV (73). Lentiviruses express two genes (*MA* and *Vpr*) that facilitate transport of viral DNA into the nucleus of nondividing cells, allowing viral integration in the absence of cell proliferation. A three-plasmid system for preparing recombinant lentiviruses consists of (*a*) a modified HIV provirus that contains defective genes for envelope proteins, (*b*) a separate plasmid containing genes encoding MLV or VSV G envelope proteins, and (*c*) a third plasmid known as a transducing vector that contains *cis*-acting sequences of HIV required for packaging, reverse transcription, and integration and a cloning casette for insertion of foreign genes (73). Thus, these vectors hold promise for stable gene transfer into cultured cells with low replicative activity. Whether recombinant lentiviruses can be grown to titers sufficient to allow metabolic studies in whole animals remains to be determined. Also, although the use of the three-plasmid system and simian rather than HIV sequences reduces concerns about safety with these vectors, replication-competent viruses still could be generated via recombination with wild-type HIV. Finally, even safer vectors, recently described, contain just 22% of the HIV genome, involving none of the pathogenic sequences (53, 122). These vectors have been used for delivery of genes to liver and muscle (53). A reporter gene was expressed in liver for more than 22 weeks, but only 3–4% of liver cells were transduced. In sum, these new vectors show promise, but current systems are somewhat complicated, some safety issues linger, and in vivo efficacy remains to be established.

Given the limitations of RNA virus vectors, increasing attention has been payed to DNA viruses for gene transfer studies, in particular herpes simplex

virus (HSV), adeno-associated virus (AAV), and adenovirus. HSV has a large genome that can accommodate more than 20 kb of insert DNA. This vector is used primarily for gene transfer to cells of the nervous system, because natural infection by HSV involves invasion of neurons or sensory ganglia and establishment of a state of latency in those cell types (39). Latent wild-type virus can be reactivated, and therefore HSV vectors are engineered to be defective in both growth and reactivation. However, even these modified vectors are toxic to neuronal cells in culture. Overall, HSV vectors have little application in nonneuronal tissues.

Like HSV or adenovirus, AAV integrates effectively into genomic DNA. Compared with RNA viruses, wild-type AAV has an advantage because it integrates at a specific site in human chromosome 19, as opposed to retroviruses whose sites of integration are random (93). Unfortunately, many AAV vectors have lost the capacity for site-specific integration and are limited to 4.8 kb or less of foreign DNA (55). Furthermore, growth of AAV requires either the presence of wild-type adenovirus, which must subsequently be removed from the viral preparation, or the use of packaging cell lines. For all these reasons, application of AAV to metabolic research has been limited, with the exception of discrete examples such as one that is discussed later.

Recombinant adenovirus has a number of attractive features for metabolic applications. First-generation adenovirus vectors are usually derived from human serotypes 2 or 5 and contain deletions in the E1A region, which controls expression of other early viral genes and is required for viral replication (16, 44). Deletion of just the E1A region allows insert sizes of up to 4.8 kb, but deletion of the nonessential E3 gene permits larger inserts. First-generation adenovirus vectors have shortcomings in terms of their immunogenicity and duration of transgene expression in vivo (see below), but they have other important positive attributes. First, they transduce a wide range of mammalian cells, including those of the liver, islets of Langerhans, muscle, and central nervous system. Second, recombinant adenoviruses can be grown at high titers with minimal effort. Third, adenoviral vectors are nearly 100% efficient in cultured cells. Fourth, adenovirus vectors effectively transduce nondividing cells, providing advantages relative to the murine leukemia based retroviruses. Finally, first-generation recombinant adenoviruses are easy to construct. Briefly, a gene or cDNA of interest is ligated into a plasmid that contains a cloning cassette with unique restriction enzyme sites interrupting the adenoviral E1A gene (43). This vector also contains a bacterial origin of replication to allow it to be amplified. This plasmid is then cotransfected with a second large plasmid that contains all of the adenovirus genome and additional "stuffer" DNA (65), rendering the viral genome too large to be packaged unless it recombines with the other plasmid. Cotransfection is usually carried out in the

human embryonal kidney cell line 293, which is an adenovirus-transformed line that provides the E1A gene product in *trans*. Homologous recombination between the two plasmids generates replication-defective recombinant adenovirus, which can be collected simply by allowing cells to lyse, recovering the resultant supernatant, and amplifying further. New recombinant viruses can be produced in 3–6 weeks (for a more detailed version of these methods, see 13).

First-generation viruses allow highly efficient gene transfer to both nondividing and dividing cells, but the viral DNA is not effectively integrated into genomic DNA (109). Gene expression is eventually lost in dividing cells because the episomal adenoviral construct becomes diluted in the population. In nondividing cells, adenovirus transferred genes are expressed for essentially unlimited periods of time (12). Adenovirus also can be used to transfer genes to the cells of intact animals. Systemic infusion of adenoviral vectors results in highly efficient and preferential gene transfer to liver cells (47, 80, 96). The preferential targeting of adenovirus-transferred genes to liver is probably explained by the sinusoidal vasculature of that organ, which allows direct contact of viral particles with hepatocytes. In other tissues, transfer is impeded by vascular barriers. In the liver of animals that receive a single systemic dose of first-generation vector, transgene expression is limited to 2–3 weeks, with some exceptions as discussed later. This is due in part to "leaky" expression of adenovirus early genes such as E2 and E4, and to activation of an immune response to these antigens (116–118). An immune response to the transgene product also can occur (106). The problem of inflammatory responses to viral antigens was initially addressed by creation of adenovirus vectors lacking specific adenovirus early genes (118). Newer, "gutless" vectors lack all viral genes (46, 60). As with lentiviral or AAV vectors, creation of these modified adenovirus vectors requires helper virus or preparation of efficient packaging cell lines. Although there have been some conflicting reports about the efficacy of these second- and third-generation vectors, duration of expression of some transgenes can be increased from 2–3 weeks to 3–12 months. Note, however, that highly efficient gene transfer to liver for a period of 2–3 weeks is sufficient to test the acute metabolic impact of many genes, and that information gathered in this way with first-generation vectors can be expanded as second- and third-generation vectors are developed.

COMPARISON OF ADENOVIRUS TO GERMLINE MANIPULATION (TRANSGENIC ANIMALS)

Relative to targeted overexpression of genes by microinjection into fertilized mouse embryos, adenovirus-mediated gene delivery has the following

advantages. (*a*) Recombinant adenoviruses can be prepared rapidly, at low cost, and can be tested in relevant in vitro systems prior to testing in animals. (*b*) Recombinant adenoviruses can be delivered to adult animals, allowing evaluation of the acute metabolic impact of a specific gene. This avoids potential compensatory or developmental effects caused by expression of a transgene throughout the life of an animal. However, with the development of inducible promoters, this advantage is becoming less important. (*c*) Germ line manipulation by microinjection of fertilized eggs with DNA has been most successfully applied in mice and is difficult in other mammals. Adenovirus-mediated gene transfer, however, can in theory be applied to any mammal and has been very successful in rats (see below). (*d*) Recombinant adenoviruses can be delivered directly to animals that serve as models of disease. For example, Zucker diabetic fatty (ZDF) rats are models of obesity and NIDDM that by classical transgenic approaches would be very difficult if not impossible to manipulate. (*e*) Adenovirus-mediated gene transfer may be more representative of eventual approaches to human gene therapy, because it is unclear whether germ line manipulation techniques will be applied to humans (40). It should also be noted, however, that perfecting safe and effective viral vectors for in vivo application in humans will require significant additional work.

There are also some important disadvantages to adenoviral vectors compared with germ line manipulation. One major disadvantage is that systemically administered adenoviral constructs are expressed primarily in liver, for reasons discussed earlier. Gene transfer can be achieved by direct injection of adenoviral vectors into extra-hepatic tissues, but expression is usually restricted to an area close to the injection site. Germ line manipulation allows expression of genes in virtually all mouse tissues, and expression of genes in a tissue-restricted fashion can be achieved with appropriate promoter/enhancer sequences in the transgene. Nevertheless, given the central role of the liver in regulation of fuel homeostasis, this limitation of adenoviral vectors is often acceptable for metabolic studies. A second major disadvantage is that adenovirus-mediated gene expression is limited in duration in vivo because animals mount immunological responses against viral gene products, the transgene product, or both (106, 116–118). Although genes introduced into fertilized eggs can be expressed throughout life, the 2- to 3-week expression window afforded by hepatic expression of a gene introduced with first-generation adenoviral vectors is often sufficient to assess its metabolic impact. Thus, important information can be obtained while improved viral vectors are being developed.

Thus, adenoviral vectors offer some distinct advantages for studies on the metabolic impact of specific genes in relevant mammalian cells. We now present specific examples of the application of adenovirus-mediated gene transfer to metabolic and nutritional research.

APPLICATION TO STUDIES OF METABOLIC CONTROL MECHANISMS

Fuel-Stimulated Insulin Secretion from β-Cells of the Pancreatic Islets

In the postprandial state, a variety of nutritional and hormonal secretagogues stimulate insulin secretion from pancreatic islet β-cells. Glucose plays a primary role, as several of the other secretagogues fail to exert their effects at low concentrations of the sugar. However, both fatty acids and certain amino acids are strong potentiators of the glucose response and are clearly important physiological regulators of insulin secretion.

Decades of investigation of the mechanism by which glucose stimulates insulin secretion has yielded a general outline of events, but much remains to be learned (Figure 1). Glucose metabolism is required because nonmetabolizable analogues such as 2-deoxyglucose or 3-O-methyl glucose are not secretagogues. Pancreatic islets contain an ATP-sensitive potassium channel, and stimulation of islets with glucose causes its closure, membrane depolarization, and activation of voltage-gated Ca^{2+} channels. The influx of Ca^{2+}, in turn, is thought to participate in regulation of protein kinases, possibly including protein kinase C and members of the Ca^{2+}/calmodulin class of kinases, to trigger exocytosis of insulin containing secretory granules. Further discussion of the experimental evidence supporting this central outline is beyond the scope of this article, but can be obtained in one of several reviews (68, 75, 76).

Within the framework outlined in Figure 1 lie a number of fundamental and unanswered questions. First, among the several ways that ATP can be generated during active glucose metabolism, is one more important or relevant than another? As summarized in Figure 1, ATP is generated in islets via the distal reactions of glycolysis and by oxidation of pyruvate in the tricarboxylic acid cycle. In addition, islets appear to contain unusually high levels of mitochondrial glycerol phosphate dehydrogenase, and they have active glycerol phosphate "shuttle" activity (62). This shuttle transfers reducing equivalents from the cytosol to the mitochondria, resulting in production of ATP via FADH and site II of the electron transport chain. It remains unclear whether all of these sources of ATP production contribute equally to regulation of the ATP-sensitive K^+ channel and insulin secretion. Second, is ATP produced as a result of glucose metabolism the only metabolic coupling signal, or are other events required? Thus, exposure of islets to stimulatory glucose suppresses fatty acid oxidation via an increase in malonyl CoA levels (25, 83) and activates phospholipases that hydrolyze phosphoinositides to produce bioactive products such as diacylglycerol and inositol trisphophate (107). However, the relevance of these events for stimulus/secretion coupling is unclear (Figure 1). Finally, in addition

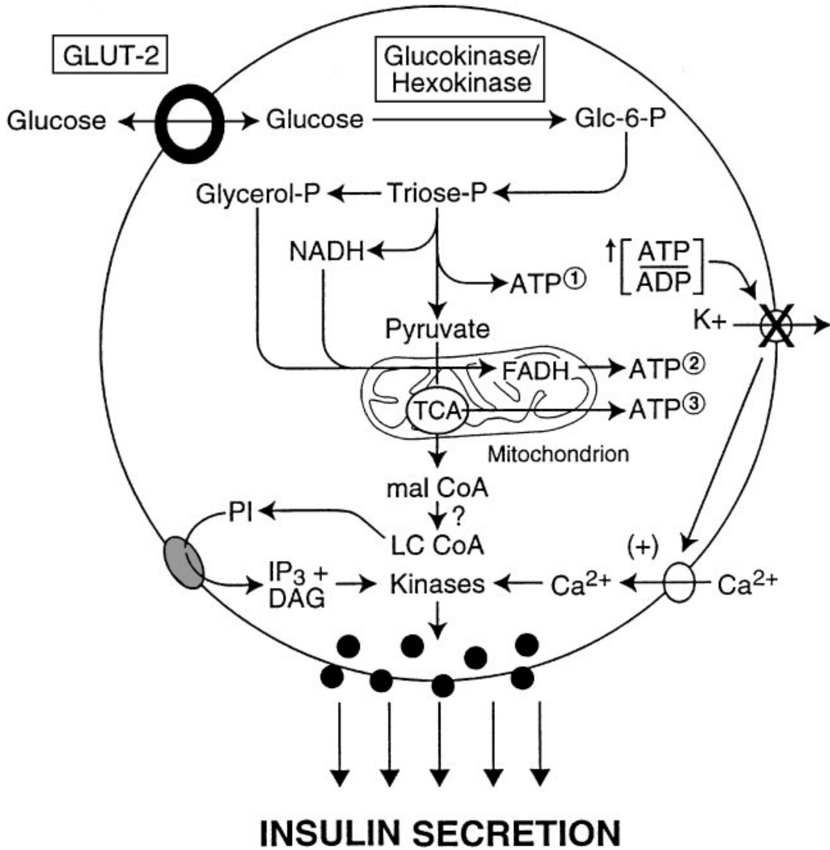

Figure 1 Biochemical coupling factors in glucose-stimulated insulin secretion. Insulin secretion is coupled to glucose metabolism in islet β-cells. Proposed coupling factors include a rise in the ATP:ADP ratio, with ATP being produced in the distal portion of glycolysis (1), via NADH shuttles (2), or via pyruvate oxidation (3). The potential linkage of glucose and lipid metabolism in regulation of insulin release is also shown.

to regulating insulin secretion in an acute fashion, glucose also increases insulin gene expression and biosynthesis of the hormone. The effects of glucose on insulin synthesis, like those on secretion, appear to require glucose metabolism, but the coupling factors may be different for the two effects. Thus, the effects of glucose on insulin secretion are measured on a timescale of seconds, whereas glucose activation of insulin biosynthesis requires minutes to hours. Also, the threshold concentration of glucose required for activation of insulin biosynthesis in islet β-cells is considerably lower than that required for stimulation of

insulin secretion (92). Further understanding of the point(s) of divergence of the two pathways is required.

ADENOVIRUS GENE TRANSFER IN ISLETS CELLS The issues raised above can be addressed by gene transfer experiments. The value of recombinant adenovirus for performing such studies was first brought to light by the demonstration that simple coculture of rat islets with a virus containing the gene for β-galactosidase results in transduction of approximately 70% of islet cells (12), considerably greater than the 10–20% transfection efficiencies for more conventional physical transfection techniques. That viruses can penetrate to the β-cell core was subsequently confirmed by immunofluoresence studies on sectioned islets (14). Since these first reports appeared, some groups have shown similarly high transduction efficiencies in rat islets (28), whereas others have demonstrated that gene transfer is mostly restricted to peripheral cells in mouse (54) or human (26) islets. The reasons for these apparent discrepancies are unknown, but it is likely that they are related to the method of islet isolation and the degree of capsular or fibrotic tissue associated with the various islet preparations. Consistent with this explanation, we have found much lower gene transfer efficiencies in islets from ZDF rats relative to our results with normal rat islets, probably because ZDF islets are much larger and more fibrotic (78). However, efficient gene transfer can be achieved in ZDF (114) or human (26) islets by perfusing the pancreas with the adenoviral preparation prior to isolating the cells. Adenoviral vectors also can be used to deliver genes with near 100% efficiency to insulinoma cell lines such as RIN 1046-38 (34) or INS-1 (4, 79). Treatment of normal rat islets (12, 14) or insulinoma cells (4, 79) with a control virus containing the bacterial β-galactosidase gene (AdCMV-βGAL) has no effect on insulin secretion or glucose or lipid metabolism relative to untreated cells, indicating that the virus per se does not interfere with the processes that we wish to analyze in these kinds of experiments.

WHICH SOURCES OF ATP ARE RELEVANT TO REGULATION OF INSULIN SECRETION? Is one source of ATP more important than another for interaction with the ATP-sensitive K^+ channel (K_{ATP}) and activation of insulin secretion? Islet β-cells do not normally metabolize glycerol or respond to it as a secretagogue; they gain these functions following adenovirus-mediated expression of glycerol kinase (79). A greater proportion of glycerol was converted to lactate and a lesser proportion was oxidized compared with glucose in these cells. The two fuels were equally potent as insulin secretagogues, despite the fact that oxidation of glycerol at its maximally effective dose (2–5 mM) occurred at a rate that was similar to that for glucose at its basal, nonstimulatory concentration (3 mM). The conclusion from these studies was that glycerol kinase expression is sufficient

to activate glycerol signaling in β-cells, which means that the failure of normal islets to respond to this substrate is due to a lack of this enzyme. Furthermore, the fact that glycerol and glucose are equally effective as secretagogues in glycerol kinase expressing INS-1 cells despite the fact that glycerol is poorly oxidized suggests that ATP produced from mitochondrial oxidation of carbohydrate secretagogues is not the primary signal for insulin secretion. Instead, the acute signal is more likely generated by the metabolism of these fuels in the glycerol phosphate shuttle and/or the distal portion of the glycolytic pathway, either of which can lead to production of ATP and an increased ATP:ADP ratio.

Other experiments have focused directly on the potential role of the glycerol phosphate shuttle in mediating glucose-stimulated insulin secretion. The activity of the mitochondrial form of glycerol phosphate dehydrogenase (GlyPDH) is reduced in islets of a variety of rodent models of NIDDM (42, 87), and in islets from humans with the disease (35). Recombinant adenoviruses containing the cDNAs encoding either the mitochondrial or cytosolic forms of GlyPDH (50) were used to treat MIN6 or HIT-T15 insulinoma cells. MIN6 cells contain levels of the two enzymes similar to those found in normal islets, and they exhibit near-normal glucose-stimulated insulin secretion. HIT-T15 is a hamster insulinoma cell line with impaired glucose responsiveness and reduced activities of both forms of the enzyme. In both MIN6 and HIT cells, overexpression of the mitochondrial form of the enzyme substantially increased the rate of conversion of 2-^{3}H glycerol to ^3H_2O, whereas overexpression of the cytosolic form had no effect on this variable. However, neither maneuver nor overexpression of both enzymes in concert had any effect on glucose-stimulated insulin secretion or the rate of glucose oxidation. The authors interpreted these findings to mean that the glycerol shuttle may not be as important for regulation of insulin secretion as was originally surmised.

More recently, the mitochondrial GlyPDH gene has been knocked out in transgenic mice, and animals homozygous for deletion of the gene (−/−) exhibit normal glucose-stimulated insulin secretion (32). In addition, animals with a genetic mutation in the cytosolic form of GlyPDH are normal (85). However, these findings do not eliminate a role for transfer of reducing equivalents from the cytosol to the mitochondria in glucose signaling, as the malate-aspartate shuttle is also very active in islet cells (62). Consistent with this hypothesis, inhibition of the malate-aspartate shuttle with aminooxyacetate (AOA) in normal islets has no effect on insulin secretion, but in islets from knock-out mice, glucose-stimulated insulin secretion is completely blocked (32). Before firm conclusions can be made, further information about the metabolic status and viability of cells with complete blockage of reducing equivalent shuttles will be required, including information about the effects of partial as opposed to complete inhibition of these shuttles.

IS NORMAL LINKAGE OF GLUCOSE AND LIPID METABOLISM REQUIRED FOR GLUCOSE-STIMULATED INSULIN SECRETION? Recent evidence suggests that glucose may also signal via a link to lipid metabolism (24, 25, 76, 83). In islets or HIT treated with glucose, malonyl CoA levels rise prior to insulin secretion. Malonyl CoA inhibits fatty acid oxidation via its effects on the mitochondrial enzyme carnitine palmitoyltransferase I (67). Because inhibition of fatty acid oxidation could cause an increase in the intracellular levels of cytosolic long-chain acyl CoA (LC-CoA), these events may be involved in regulation of insulin secretion, an idea now known as the LC-CoA hypothesis (25, 83). It is clear that some minimal pool of intracellular lipid is required for normal islet function. Thus, administration of nicotinic acid (94, 95) and hyperleptinemia created by infusion into normal rats of a recombinant adenovirus containing the leptin cDNA (58) (see below for further discussion of this model) cause depletion of tissue lipid stores (complete depletion of all islet triglycerides in the case of the hyperleptinemic model). These treatments also block insulin secretion in response to a variety of secretagogues, including glucose. Full secretory function was restored in these lipid-depleted animals by providing fatty acids to the pancreas (58, 94, 95). Fatty acids also acutely potentiate glucose-stimulated insulin secretion (15, 97). Feasible sites at which increased intracellular lipids could influence insulin secretion include conversion to bioactive metabolites such as diacylglycerol or inositol trisphosphate (IP_3), contribution to plasma membrane or secretory granule membrane lipid turnover, or direct acylation of proteins involved in secretory granule trafficking.

To evaluate the LC-CoA hypothesis, INS-1 insulinoma cells were treated with a recombinant adenovirus containing the cDNA encoding malonyl CoA decarboxylase (AdCMV-MCD) (4). The resultant increase in decarboxylation of malonyl CoA to acetyl CoA caused a dramatic decrease in intracellular malonyl CoA levels compared with cells treated with the β-galactosidase control virus at both 3 and 20 mM glucose. Furthermore, in AdCMV-MCD–treated cells, 20 mM glucose was less effective at suppressing [1-^{14}C]palmitate oxidation. AdCMV-MCD–treated cells also incorporated 43% less labeled palmitate and 50% less labeled glucose into cellular lipids than did control cells. It is important to note that the rate of [5-^{3}H]glucose utilization or [1-^{14}C]glucose oxidation was unchanged in MCD-overexpressing cells relative to controls, as was glucose-stimulated insulin secretion. These studies demonstrate that genes delivered by recombinant adenovirus can be used to disrupt discrete metabolic events, leaving some other metabolic pathways intact and unaltered.

These findings were corroborated by use of a pharmacologic agent, triacsin C, an inhibitor of LC-CoA synthase (4). Administration of the drug to INS-1 cells caused attenuation of palmitate oxidation, a reduction in glucose or palmitate incorporation into cellular lipids, and a 47% decrease in total LC-CoA levels, but no change in insulin secretion in response to glucose. Triacsin C

also was applied to freshly isolated rat islets of Langerhans, where it caused the expected suppression of fatty acid oxidation but, as in INS-1 cells, had no effect on glucose-stimulated insulin secretion. Finally, the drug was tested in INS-1 cells engineered for glycerol kinase expression. Incorporation of radiolabeled glycerol into cellular lipids was effectively blocked by triacsin C (presumably because of depletion of LC-CoA required for esterification to glycerol phosphate), but the drug had no effect on glycerol-stimulated insulin secretion (79).

The foregoing studies show that significant impairment of the link between glucose and lipid metabolism in β-cells is tolerated with no impact on glucose-stimulated insulin secretion, as long as glycolytic flux is not perturbed. With the apparent elimination of changes in malonyl CoA levels and attendant alterations in lipid metabolism, the search for coupling factors for glucose-stimulated insulin secretion can be narrowed. These experiments, like those with the glycerol kinase virus, call particular attention to immediate byproducts of glucose catabolism rather than to a requirement for conversion of glucose into other classes of metabolites. However, we do not mean to imply that lipids have no role in regulation of insulin secretion. Note that islets depleted of lipid as a result of leptin or nicotinic acid treatment not only fail to respond to glucose, but also show no response to arginine, leucine, or the sulfonylurea, glibenclamide (29, 58). Arginine is thought to exert its secretory effects by directly affecting membrane polarization, whereas sulfonylureas are believed to bring about the same effect by inhibiting ATP-sensitive K^+ channel activity. Thus, both agents act distal to any anticipated early metabolic signal. With all secretagogues tested, the attenuated insulin response in lipid-depleted islets is restored to normal by fatty acids (29, 58, 94, 95). The mechanism by which fatty acids exert these important modulatory effects on insulin secretion remains to be established.

Metabolic Control Mechanisms in Liver Cells

The mammalian liver plays a critical role in fuel homeostasis. In the fasted state, hepatic glucose production is activated by concurrent increases in gluconeogenesis and glycogenolysis, thereby providing protection against hypoglycemia. In fasted animals, levels of circulating free fatty acids begin to rise because of increased lipolysis in adipose tissue. This rise is modulated by increased uptake and oxidation of fatty acids in liver. In the transition from fasted to refed state, the liver begins to store glucose in the form of glycogen and becomes a producer of fat in the form of very-low-density lipoproteins (VLDL). These fundamental functions of liver are profoundly disturbed in obesity, insulin resistance, and NIDDM. In individuals with NIDDM, ingestion of a meal, even one containing carbohydrates, fails to exert its normal suppressive effect on hepatic glucose output, and it also fails to trigger the normal surge in glucose disposal and storage. NIDDM patients store less glycogen in their livers than do normal subjects, and as a consequence of peripheral insulin resistance and

sustained VLDL production, they are hyperlipemic. In this section, we review the use of adenovirus-mediated gene transfer to investigate metabolic control mechanisms in the liver.

THE ROLE OF GLUCOSE PHOSPHORYLATING ENZYMES In terms of the glucose disposal and storage function of liver, a great deal of attention has been payed to the role of glucokinase (GK) [hexokinase (HK)-IV]. GK has a lower affinity for glucose and a higher catalytic capacity than do other members of its gene family. It is limited in terms of tissue distribution to liver, the islets of Langerhans, and certain specialized neuroendocrine cells in the pituitary and gastrointestinal tract (49, 51). Liver and islet β-cells also express a specialized facilitated glucose transporter, GLUT-2, which like GK is distinguishable from other members of its gene family in terms of its lower affinity for glucose and high transport capacity. Thus, GLUT-2 and GK serve to regulate entry of glucose into pathways of glucose disposal and storage in liver and islet β-cells (74).

The balance between hepatic glucose production and glucose disposal on the one hand and glucose disposal and storage on the other is determined by the relative rates of glucose phosphorylation and glucose-6-phosphate (G6P) hydrolysis (Figure 2). G6P hydrolysis is mediated by the glucose-6-phosphatase enzyme complex, comprised of a catalytic subunit sequestered as an integral membrane protein of the endoplasmic reticulum (ER), a G6P translocase known as T1 that delivers G6P to the catalytic subunit, and putative ER glucose and inorganic phosphate transporters that move the reaction products back into the cytosol (7, 81) (Figure 2). Expression of glucose-6-phosphatase is increased in animal models of NIDDM (11, 105) and IDDM (61), and this increase may contribute to elevated hepatic glucose production in both cases (see also below). This raises the possibility that improved glucose disposal might be achieved by increasing the expression of GK or decreasing glucose-6-phosphatase.

The first evidence in support of this hypothesis came from studies of stable transfection of hepatoma cell lines, FTO-2B and H4IIE (108). Both cell types lack endogenous GK and have very low capacities for glycogen synthesis. Expression of GK in these cells causes a large increase in glycogen synthesis and glycolytic flux, consistent with the idea that GK can regulate glucose disposal in hepatocyte-derived cell lines. Because both cell lines lack endogenous GK, these results do not predict the metabolic impact of overexpression of GK or any other HK isoform in primary hepatocytes with a normal complement of GK activity. Additionally, the levels of the GK regulatory protein in hepatoma cell lines is unknown. This protein is proposed to play a role in the acute regulation of GK activity (110).

To address these issues, recombinant adenovirus was used to compare the metabolic impact of overexpression of GK with that of overexpressed HK-I in primary rat hepatocytes (82). HK-I has a much lower K_m for glucose than does

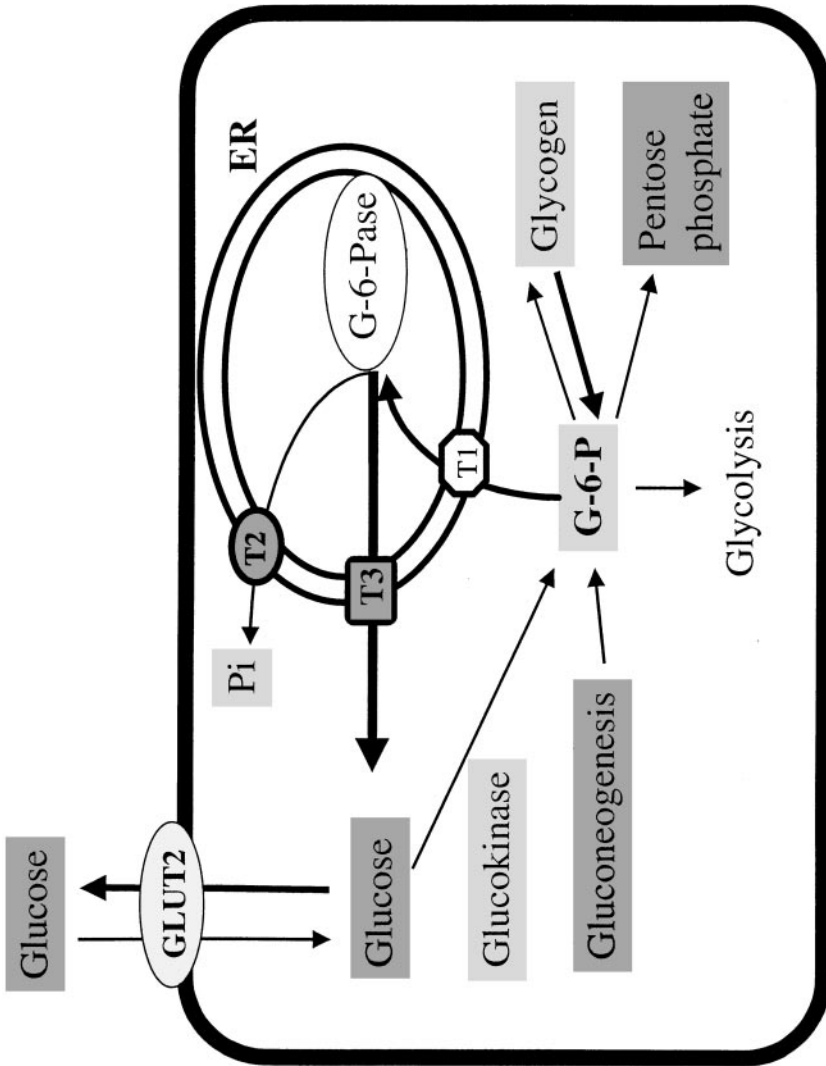

Figure 2 Regulation of glucose disposal in liver by the balance between glucose phosphorylation and hydrolysis of glucose-6-phosphate (G-6-P). The glucose-6-phosphatase complex is shown, consisting of a G6P translocase known as T1, a catalytic phosphohydrolase (G-6-Pase), and putative transporters for inorganic phosphate (Pi, T2) and glucose (T3). The complex is localized to the endoplasmic reticulum (ER).

GK (50 μM versus 8 mM, respectively). In addition, HK-I is allosterically inhibited by G6P whereas GK is not (115). AdCMV-GK and AdCMV-HK-I viruses were applied to cultured rat hepatocytes at titers that, in each case, caused an approximate sevenfold increase in total glucose phosphorylation capacity. AdCMV-GK–treated hepatocytes incubated for 48 h in the presence of variable glucose concentrations had glycogen levels that were maximally 15-fold greater than levels in corresponding control cells, whereas AdCMV-HK-I–treated hepatocytes incubated under similar conditions exhibited no increase in glycogen content. Similarly, AdCMV-GK treatment caused a 3-fold increase in lactate output and a 3.5-fold increase in glucose oxidation, whereas treatment with AdCMV-HK-I caused only a modest increase in lactate output, and only at low glucose concentrations. In sum, overexpression of GK stimulated large increases in glucose storage and utilization in hepatocytes, whereas overexpression of HK-I did not.

We next investigated the mechanism underlying the striking differential effects of the two HK isoforms on glycogen synthesis (88). G6P levels increased approximately fourfold in both AdCMV-GK– and AdCMV-HK-I–treated hepatocytes compared with untreated cells, as glucose was raised from 1 to 5 mM. When the glucose concentration was raised above 5 mM, no further increase in G6P concentration was observed in AdCMV-HK-I–treated cells, whereas it continued to accumulate in AdCMV-GK–treated cells, reaching a level more than 50 times higher than basal. UDP-glucose accumulated in parallel with G6P in both AdCMV-HK-I– and AdCMV-GK–treated cells. Despite identical increases in G6P and UDP-glucose achieved in GK and HK-I overexpressing cells at low glucose concentrations (1–5 mM), only AdCMV-GK transfected cells exhibited an increase in the activity state of glycogen synthase. These studies indicate that G6P produced by overexpressed GK is glycogenic because it effectively promotes activation of glycogen synthase. G6P produced by overexpressed HK, in contrast, did not exert the same regulatory effects, possibly because GK and HK-I have different subcellular distributions. Consistent with this interpretation, a large portion of overexpressed HK-I was shown to be associated with a mitochondria-enriched fraction, whereas none of the overexpressed GK was in this fraction (82).

New information about subcellular localization of GK under different nutritional conditions provides context for the foregoing observations. GK regulatory protein (GRP) binds to and inhibits GK in a hexose phosphate–regulated manner (110). GRP sequesters GK in the hepatocyte nucleus in the presence of low glucose and insulin concentrations, as in the fasted state (19, 103). Increases in external nutrients such as glucose or fructose stimulate translocation of GK from the nucleus to the cytosol, with no measurable change in the subcellular distribution of low K_m HKs (1, 19, 103). Graded overexpression of GK with recombinant adenovirus and digitonin permeabilization of hepatocytes

was used to measure free (soluble) and bound GK activities (2). Binding of GK in digitonin permeabilized cells (presumably to GRP) was saturable, and the rate of glycogen synthesis was correlated with free rather than bound GK activity. A similar nutrient-regulated translocation of glycogen synthase has also been reported (33, 38).

The model that emerges from all of these results is summarized in Figure 3. At low glucose concentrations, as in the fasted state, GK is sequestered in the nucleus bound to GRP, and glycogen synthase is in a perinuclear (liver) or nuclear (muscle) compartment. On stimulation with nutrients, GK is translocated out of the nucleus, and glycogen synthase moves toward the plasma membrane of the cell. Consistent with this model, recent electron microscopy studies reveal that in liver of fasted-refed rats, glycogen particles first appear at the plasma membrane, with subsequent synthesis occurring in a gradient from the exterior toward the interior of the cell (36). This model suggests an explanation for the differential metabolic impact of overexpressed GK and HK-I. GK and glycogen synthase, both of which are translocated toward the periphery of the cell by increased nutrient supply, may become juxtaposed such that G6P produced in the GK reaction has ready access to glycogen synthase, stimulating its activity. In contrast, HK does not undergo the same translocation (the more active enzyme remains associated with mitochondria), and as a consequence, G6P produced by this enzyme does not have the same impact on glycogen synthesis. Although this model fits well with current data, there is no direct evidence for a close association of GK and glycogen synthase during nutrient stimulation of hepatocytes. Further discussion of the importance of protein/protein interactions in the regulation of glycogen synthesis is provided below.

GLUCOKINASE OVEREXPRESSION REVEALS FUNDAMENTAL METABOLIC DIFFERENCES BETWEEN LIVER AND ISLET CELL It has long been appreciated that GK plays an important role in regulation of glucose flux in islet β-cells (68, 74–76). Genetic deficiencies in the enzyme have been linked to impaired insulin secretion in a form of NIDDM called maturity-onset diabetes of the young (41). Furthermore, experimental reduction in GK expression in liver or islets in transgenic mice causes reduced glucose-stimulated insulin secretion and less-efficient suppression of hepatic glucose output during a glucose clamp (9, 45, 101). Based on these studies of the effects of underexpression of GK, one might have predicted that the overexpression of the enzyme should provide an enhancement of glucose utilization in islet cells similar to that observed in the experiments described earlier with hepatocytes. Surprisingly, adenovirus-mediated overexpression of GK in isolated rat islets did not increase glucose usage, lactate production, or glycogen synthesis, whereas overexpression of HK-I did increase glucose usage and insulin secretion at low glucose concentrations (12, 14).

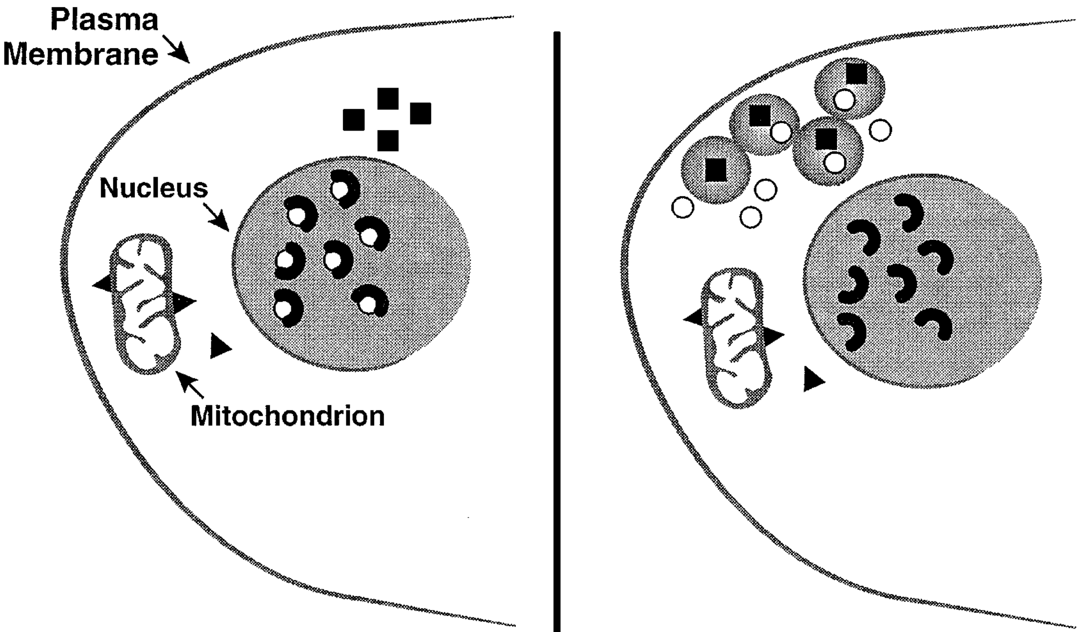

Figure 3 Regulation of glycogen metabolism by nutrient-mediated translocation of relevant enzymes. In the fasted state, glucokinase is sequestered in the nucleus of liver cells by binding to the glucokinase regulatory protein. Upon stimulation with glucose or other sugars, glucokinase is translocated out of the nucleus, perhaps joining glycogen synthase as it moves toward the plasma membrane. Hexokinase, in contrast, is not translocated by nutrients and remains associated with mitochondria.

We compared the impact of overexpressed GK in the well-differentiated insulinoma cell line, INS-1, and rat hepatocytes (17). By applying different titers of recombinant adenovirus, a range of overexpression of GK from two- to tenfold was achieved in INS-1 cells. Over this range, there was a progressive increase in [2-^{3}H]- or [5-^{3}H]glucose usage at low ($\leq$5 mM) but not high glucose concentrations, whereas glucose usage was increased at both low and high glucose concentrations in similarly treated hepatocytes. Furthermore, utilization of [2-^{3}H]glucose in INS-1 cells was suppressed in GK overexpressing INS-1 cells by high glucose in a rapid and reversible fashion, whereas such regulation was largely absent in hepatocytes. Levels of hexose phosphates (G6P, fructose-6-phosphate, and fructose-1,6-bisphosphate) were profoundly and rapidly elevated following a switch from low to high glucose in GK overexpressing INS-1 cells or hepatocytes relative to controls. In contrast, triose phosphate levels (glyceraldehyde-3-phosphate + dihydroxyacetone phosphate) were much higher in AdCMV-GK–treated INS-1 cells than in similarly treated hepatocytes. These results suggest that flux through the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) step is slower in the former cells. Islets or INS-1 cells contain much less lactate dehydrogenase activity than do hepatocytes (17, 86). Consistent with this, the pyruvate:lactate ratio in AdCMV-GK–treated INS-1 cells is much higher than that in similarly treated hepatocytes (17). Because the activities of G3PDH in INS-1 and hepatocyte extracts were similar, flux through this step in INS-1 cells may limit the rate of regeneration of NAD in the lactate dehydrogenase reaction. Thus, a fundamental difference between hepatocytes and islet β-cells revealed by these studies is the limited capacity of the latter to metabolize glycolytic intermediates beyond the G3PDH step.

These findings are generally consistent with those of another group who studied INS-1 cells stably transfected with a tetracycline regulatable GK construct (111). Twofold overexpression of GK caused a proportional increase in [5-^{3}H]glucose usage at both low and high glucose, but further increases in expression resulted in little additional increase in glycolysis at higher glucose concentrations. High levels of GK overexpression in INS-1 cells were subsequently shown to cause ATP depletion, and based on this, the failure to see enhanced glycolytic flux at these high enzyme levels was interpreted to be due to general metabolic paralysis (112). However, in our studies, GK overexpression resulted in similarly low intracellular ATP concentrations in INS-1 cells and hepatocytes (17). More extensive biochemical analysis will be required to fully resolve the role of fluctuations in ATP levels in mediating the metabolic impact of GK overexpression.

These studies suggest that increasing the expression levels of GK in liver may be a powerful way to lower circulating glucose levels in diabetic subjects. Consistent with this, transgenic mice with GK overexpressed in liver under

control of the PEPCK promoter and also rendered diabetic by streptozotocin injection are able to partially control hyperglycemia (37). This potentially beneficial impact of GK overexpression must be weighed against the potential for perturbation of lipid homeostasis, as increased glycolytic flux could lead to increased de novo lipogenesis and VLDL production. This is a particular concern in NIDDM, a disease in which hyperlipidemia is already present and could become exacerbated. More in-depth testing of the effect of hepatic GK overexpression on glucose and lipid homeostasis is called for, particularly in animal models of NIDDM. Because many of these models are difficult to access by germ line manipulation, this is an area in which recombinant adenovirus vectors can provide important insights.

On the other hand, studies discussed above suggest that overexpression of GK in islet β-cells may have limited therapeutic value because of constraints on metabolic impact imposed by distal reactions of the glycolytic pathway. Surprisingly, in vivo confirmation of this idea from transgenic animal studies is not available, because there has been no report on the effect of GK overexpression specifically in islets. In a transgenic model involving an 80-kb fragment of the GK gene directing expression of both the hepatic and β-cell isoforms, GK expression was actually decreased in β-cells, apparently as a secondary consequence of enhanced hepatic glucose disposal caused by overexpression of the enzyme in the liver (77). Further insights are unlikely to be gained with currently available adenoviral vectors, as they are not useful for delivering genes to islets of intact animals (78, 80).

The limited metabolic impact of GK overexpression may be a relatively specific feature of β-cells, as adenovirus has also been used to overexpress the enzyme in isolated human myocytes, resulting in potent increases in glucose storage and utilization (10). This maneuver also increased the concentration of glucose required to activate glucose metabolism, consistent with the idea that the high K_m GK assumes a controlling role relative to the endogenous low K_m HK-II of muscle cells. Whether expression of GK in muscle of animals or humans with NIDDM will have a therapeutic effect remains to be determined.

TESTING OF NEWLY DISCOVERED GENES AND DISEASE CANDIDATE GENES

Phosphatase Targeting Proteins and Compartmental Control of Glycogen Metabolism

GK was discovered 40 years ago (21), so it is remarkable that not until recently has a full understanding of its role in control of glucose metabolism in important tissues been developed. Application of high-efficiency gene transfer

technologies, including recombinant adenovirus, has increased the pace at which we unravel the functions of new proteins with potential metabolic impact. For example, protein targeting to glycogen (PTG, also known as PPP1R5) is a recently discovered gene (31, 84) and a member of a family of glycogen targeting subunits of protein phosphatase-1 (PP-1) (8). These proteins target PP-1, the enzyme responsible for activation of glycogen synthase and inactivation of glycogen phosphorylase and phosphorylase kinase (48), to the glycogen particle. They also appear to participate in assembly and regulation of the enzymes of glycogen metabolism within mammalian cells. Thus, in addition to its capacity to bind PP-1 and the glycogen particle, PTG appears to bind directly to glycogen synthase and phosphorylase kinase (31, 84).

Four members of the family have been identified, and the structural relationships among the three most-studied family members are summarized schematically in Figure 4. A 124-kDa form known as G_m or RG1 is expressed preferentially in heart and skeletal muscle (99). Human G_M is phosphorylated on serine-46 in response to insulin, which appears to enhance the capacity of the G_M–PP-1 complex to dephosphorylate and activate glycogen synthase, whereas β-adrenergic agonist-mediated phosphorylation of serine-63 (serine-65 in the rabbit enzyme) results in dissociation and inactivation of the G_M–PP-1 complex (27). Another form, G_L, is a 33-kDa protein that is expressed primarily in liver and appears to be regulated allosterically by binding to phosphorylase a (30). G_L lacks serine-46, and although it contains a serine at position 61 (analogous to serine 63 in G_M), it appears not to be regulated by a phosphorylation/dephosphorylation mechanism (30). The recently discovered 36.4-kDa PTG form also activates glycogen synthesis in a hormone-independent fashion, but it is distinct from G_L in that it has a wide tissue distribution and no apparent regulation by phosphorylase a (31, 84). Finally, another form known as PPP1R6 is similar to PTG in its wide distribution and its apparently unregulated interaction with PP-1 (8).

Characterization of these newly cloned glycogen targeting subunits, including their interactions with various enzymes of glycogen metabolism, has advanced rapidly, but little is known about the metabolic impact of these proteins. What are the functions of these distinct forms of glycogen targeting subunits, and why do they have different tissue distributions and regulatory properties? To begin to address these issues, recombinant adenovirus was used to overexpress PTG in primary rat hepatocytes (18), allowing evaluation of its metabolic impact in a cell type with a large capacity for glycogenesis. Overexpression of PTG in hepatocytes isolated from fasted rats resulted in potent activation of glycogen synthesis, such that levels of glycogen in AdCMV-PTG–treated cells approximated those in the liver of fed animals. The glycogenic effect of PTG did not require carbohydrate or insulin in the culture medium. Thus, the same amount

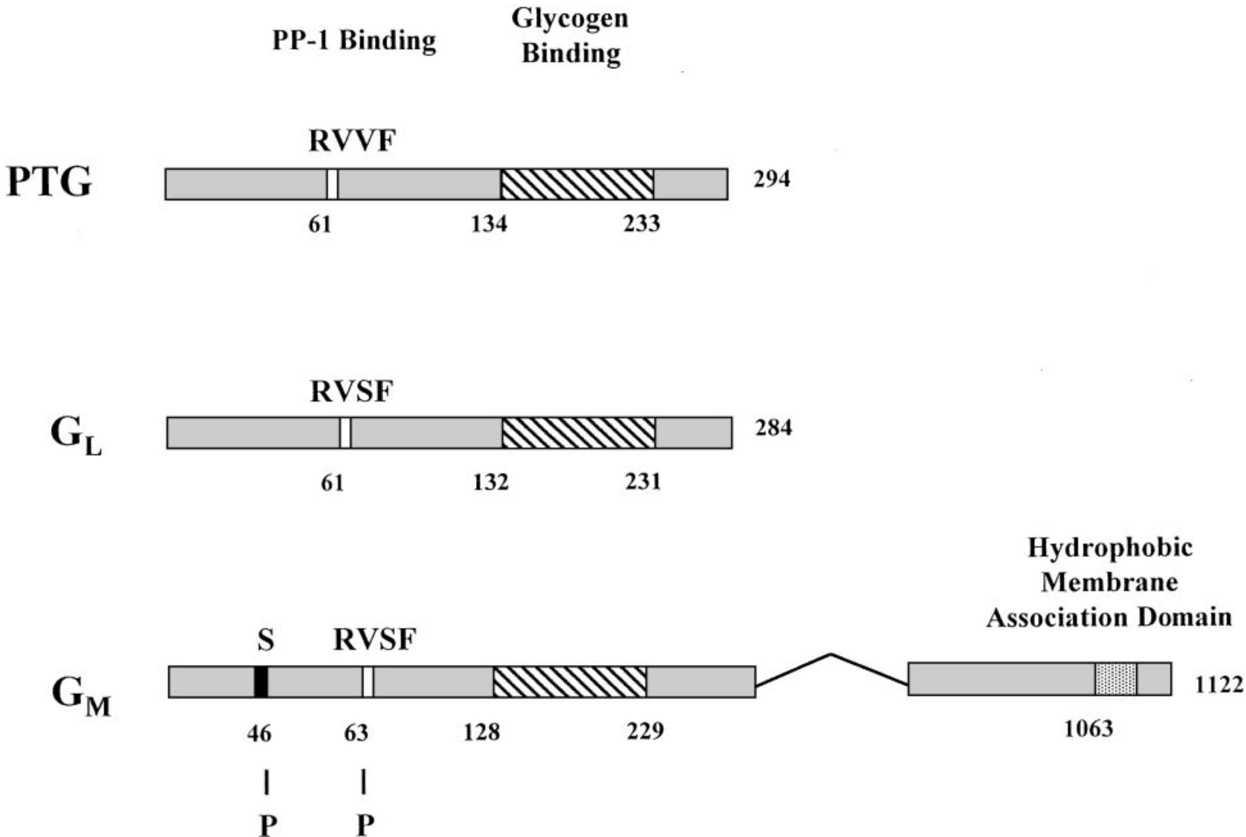

Figure 4 Key members of the family of proteins that target protein phosphatase-1 (PP-1) to the glycogen particle. The schematic alignment of protein sequences of protein targeting to glycogen (PTG) (expressed in many tissues), G_L (expressed primarily in liver), and G_M (expressed primarily in muscle) shows putative PP-1 binding domains, glycogen binding domains, and serine residues that appear to be phosphorylated in G_M but not in the other isoforms.

of glycogen was synthesized in PTG-overexpressing cells cultured in HEPES buffer with glutamine but without glucose, as in complete tissue culture medium supplemented with glucose (18). PTG overexpression also prevented the normal glycogenolytic action of forskolin or glucagon. These metabolic effects of PTG overexpression were accompanied and likely explained by a 3.6-fold increase in glycogen synthase activation state, and a 40% decrease in glycogen phosphorylase activity. These results are consistent with a model in which PTG overexpression "locks" the hepatocyte in a glycogenic mode, presumably via its ability to promote interaction of enzymes of glycogen metabolism with PP-1.

These in vitro studies suggest that changes in the expression levels of PTG or its family members may have relevance for treatment of diabetes. A fundamental metabolic defect of NIDDM is the failure to suppress hepatic glucose output in the fed state, and this is reflected in lower levels of hepatic glycogen in individuals with the disease (63). Up regulation of expression of glycogen targeting subunit proteins in liver by pharmaceutical or genetic methods may enhance glucose disposal and glycogen storage in NIDDM subjects. However, PTG may not be the optimal form of glycogen-targeting subunit for manipulation, because it appears to override normal hormonal control mechanisms for glycogen metabolism. Expression of other family members, such as G_M or G_L, in contrast, may stimulate glucose disposal while allowing substrate and hormone-mediated regulation of glycogen turnover. Construction of recombinant adenoviruses containing the genes for G_M and G_L should allow rapid testing of this idea.

Glucose-6-Phosphatase as a Diabetes Candidate Gene

The terminal step of gluconeogenesis is the hydrolysis of G6P to free glucose, catalyzed by the glucose-6-phosphatase enzyme complex (described above) (see Figure 2). Increased activity of the glucose-6-phosphatase complex has been implicated in the dysfunction of both β-cells and hepatocytes in NIDDM. In islets of ob/ob mice, a model of obesity and NIDDM, the rate of glucose recycling (glucose $\rightarrow\rightarrow$ G6P $\rightarrow\rightarrow$ glucose) is greatly increased relative to that in islets from normal lean animals (56, 57). Glucose-stimulated insulin secretion is diminished in this and other genetic models of obesity and NIDDM, and excessive glucose recycling could contribute to the defect by causing increased consumption of ATP (102). These studies implied that the molecular basis for increased glucose cycling in islets from ob/ob mice is increased glucose-6-phosphatase activity, but this variable was not measured. More recently, a survey of a large number of mRNAs in pancreatic islets revealed that the mRNA encoding the catalytic subunit of glucose-6-phosphatase (G6Pase) is increased by 43% in islets of prediabetic and by more than fivefold in diabetic ZDF animals relative to lean ZDF controls (102). G6Pase was one of only two islet mRNAs

among the 31 surveyed whose abundance was increased in the obese and diabetic animals relative to control animals. Like islets from ob/ob mice, those from ZDF rats have elevated rates of glucose usage and impaired regulation of insulin secretion (52, 69, 102).

These studies suggest that increased expression of G6Pase may be involved in β-cell dysfunction of obesity and NIDDM. However, it must be remembered that G6P hydrolysis is catalyzed by a complex of proteins (see Figure 2). Thus, if one of the other components of the system (i.e. the T1 translocase or the glucose or inorganic phosphate transporters) is rate limiting, increased expression of the catalytic subunit alone should have limited impact on the overall rate of G6P hydrolysis. To address this issue directly, recombinant adenovirus was used to overexpress G6Pase in INS-1 cells (104). The overexpressed catalytic subunit was normally glycosylated, correctly sorted to the endoplasmic reticulum, and caused a tenfold increase in enzymatic activity in in vitro assays. It also caused a fourfold increase in ^3H_2O incorporation into glucose in INS-1 cells, indicative of enhanced G6P hydrolysis. Overexpression of the catalytic subunit also caused a 32% decrease in glycolytic flux ([3-^{3}H]glucose usage) relative to controls and a proportional decrease in glucose-stimulated insulin secretion. These studies show that overexpression of G6Pase alone significantly impacts glucose metabolism and insulin secretion in islet β-cells, without a requirement for overexpression of other components of the hydrolytic complex. However, INS-1 cells engineered for overexpression of the catalytic subunit do not exhibit the severe alterations of β-cell function and metabolism associated with islets from rodent models of obesity and NIDDM, which suggests the involvement of genes in addition to G6Pase in the etiology of such β-cell dysfunction.

Increased expression of G6Pase also could be involved in loss of control of hepatic glucose production characteristic of NIDDM. In fact, there is increased expression of G6Pase in liver of rodent models of both type I and type II diabetes (11, 61, 105). In ZDF (−/−) rats, an approximate 2.5-fold increase in expression of the catalytic subunit was correlated with a similar increase in enzyme activity in hepatic microsomes relative to samples from lean ZDF (+/+) or normal Wistar control rats (105). Furthermore, hyperglycemia and hyperlipidemia cause increased expression of hepatic G6Pase both in cultured cells and in whole animals (6, 64, 70). Linkages between mutations in the gene encoding G6Pase and human NIDDM have not been established. Such an analysis is complicated because the anticipated mutation is likely to increase rather than decrease expression of the enzyme, and it may not involve mutations in the protein-coding sequence of the G6Pase gene. While the search for such genetic mutations continues, further insight into the potential role of G6Pase in the etiology of NIDDM may be gained by studies in which the enzyme is overexpressed in liver.

To this end, recombinant adenovirus has been used to overexpress G6Pase in isolated hepatocytes and in liver of normal animals (89, 105). Similar to INS-1 cells, overexpression of G6Pase in rat hepatocytes caused an eightfold increase in G6P hydrolysis, as measured by incorporation of ^3H_2O into medium glucose, and an attendant 25% reduction in intracellular G6P levels (89). Overexpression of the enzyme in hepatocytes also caused a 45% increase in gluconeogenesis, a 32% decrease in [^{3}H]glucose usage, and a 55% decrease in glycogen levels relative to untreated or AdCMV-βGAL–treated control cells. These studies demonstrate that increased expression of G6Pase in liver cells has the potential to perturb fuel homeostasis in whole animals.

To futher investigate this point, a recombinant adenovirus encoding G6Pase was infused into normal Wistar rats via the tail vein (105). This maneuver resulted in a two- to threefold increase in the level of the catalytic subunit in liver, accompanied by a similar increase in enzyme activity in hepatic microsomes, relative to animals infused with a control virus. It is important that no increase in expression of the transgene was observed in extra-hepatic tissues such as muscle, adipose, or pancreas, which is evidence for preferential uptake of systemically administered adenoviral vectors in the liver of rats, consistent with previous observations in mice (47). AdCMV-G6Pase–infused rats exhibited several of the abnormalities associated with early stage NIDDM, including glucose intolerance, hyperinsulinemia, decreased hepatic glycogen content, and increased peripheral (muscle) triglyceride stores. These animals also exhibited significant decreases in circulating free fatty acids and triglycerides, changes not normally associated with the disease.

These findings are consistent with the idea that increased expression of G6Pase in liver can make a significant contribution to the development of NIDDM. G6Pase mutations may be the primary genetic lesion in some individuals with the disease. In this scenario, a gradual increase in the degree of overexpression of G6Pase would result in development of glucose intolerance and hyperinsulinemia, resulting in turn in increased lipid storage in peripheral tissues. Peripheral overstorage of fat is strongly correlated with development of insulin resistance (reviewed in 66). If excess lipid storage actually causes insulin resistance, long-term overexpression of G6Pase in the liver may cause the gradual development of hyperlipidemia and exacerbation of glucose intolerance, complementing hyperinsulinemia, and reduced hepatic glycogen levels to produce the entire syndrome of NIDDM. Alternatively, overexpression of G6Pase in liver could be secondary to increases in circulating glucose and lipid levels that occur in response to genetic lesions in insulin signaling or β-cell function rather than in the G6Pase gene itself. Finally, intermediate models, in which G6Pase interacts in different ways with other candidate genes that predispose to insulin resistance or β-cell failure, are also possible. Such a

hypothesis is consistent with recent examples of interactions of genes in the development of diabetes. Thus, breeding of mice heterozygous for knocking out the insulin receptor substrate-1 (IRS-1) with mice heterozygous for knocking out other candidate genes such as β-cell GK or the insulin receptor cause diabetes, whereas any of these mutant strains studied individually are normoglycemic (5, 20, 98, 100). Adenovirus-mediated gene delivery to the livers of these various transgenic strains may provide new insights into the potential interaction of G6Pase overexpression with other genetic factors predisposing to NIDDM.

DELIVERY OF PEPTIDE HORMONES

The levels of peptide hormones or other proteins in the circulation of intact animals can be controlled by direct injection of adenovirus or other viral vectors into tissues such as skeletal muscle, or by their systemic infusion, in which case the transgene will be expressed primarily in liver. Expression of transgenes introduced in this manner can be controlled by constitutive promoters, by promoters that are known to be regulated in the target tissue (i.e. PEPCK promoter in liver), or by the use of a promoter that can be induced by addition of a drug or small molecule (i.e. tetracycline-regulated systems). It should be noted that these approaches are not capable of reproducing tightly regulated delivery of a hormone such as insulin, for which metabolic signaling mechanisms and the secretory apparatus of neuroendocrine cells are required. Nevertheless, a great deal can be learned about the metabolic impact of newly emerging hormones by causing sustained increases in systemic levels in whole animals. The example discussed here is the use of adenovirus to create experimental hyperleptinemia in rodents.

Leptin is a peptide hormone produced in adipocytes that plays a major role in control of food intake and thermogenesis (119). It was discovered as the mutated gene in ob/ob mice, a genetic model of obesity and NIDDM. Infusion of the hormone causes a decrease in food intake and depletes fat stores, making it a potential therapeutic agent for human obesity and diabetes. However, early clinical trials conducted by infusion of recombinant leptin into obese humans have shown modest effects. Factors influencing the efficacy of injection therapy with leptin include the apparent leptin resistance of obese subjects (22), inflammatory responses that develop at the injection site, and the inherent difficulty in maintaining sustained increases in circulating levels of a hormone with a short half-life.

Several groups have used viral vectors for systemic delivery of leptin. In the first of these studies, recombinant adenovirus vectors were used to deliver the hormone to normal rats (23) or ob/ob mice (72). In normal rats, systemic

infusion of a virus containing the rat leptin cDNA resulted in expression of leptin in liver. Constitutive release of the hormone into the blood caused a 10-fold increase in levels of the hormone for the full 28-day term of the study relative to animals that received an infusion of saline or a control virus (23). Hyperleptinemic rats exhibited a 30–50% reduction in food intake and gained only 22 g over the period of the experiment versus 115–132 g gained by control animals. Body fat was completely ablated in the hyperleptinemic animals, with no apparent side effects (animals appeared healthy and were normally vigorous). Although animals were initially examined at the end of the 28-day experiment, in more recent studies leptin-induced ablation of fat occurred within 5 days of administration of the leptin virus (59). Animals pair-fed to the amount of food ingested by the hyperleptinemic rats had the same low rate of weight gain but had only a partial reduction in body fat. Furthermore, plasma triglyceride and insulin levels were significantly lower in hyperleptinemic versus pair-fed animals, whereas free fatty acid and glucose levels were similar in the two groups, suggestive of enhanced insulin sensitivity in the hyperleptinemic animals. Thus, despite equivalent reductions in food intake and weight gain in hyperleptinemic and pair-fed animals, identifiable fat tissue was completely ablated only in the former group, confirming that leptin has metabolic effects independent of its effects on food intake. Whereas the pair-fed normal group exhibited a rise in circulating ketones (as would normally occur in food-restricted or fasted animals), this did not occur in hyperleptinemic animals. One possible explanation is that leptin may induce an increase in fatty acid oxidation in adipocytes (90). A series of subsequent studies has demonstrated direct actions of leptin to induce fatty acid oxidation and inhibit esterification in pancreatic islets (90, 91, 113, 114, 120, 121). The extent to which these direct actions of leptin are involved in its physiological effects remains to be determined.

Recombinant adenovirus has also been used to deliver leptin to ob/ob mice (72). These animals contain a point mutation in the leptin gene that introduces a premature stop codon, resulting in production of a truncated, biologically inactive form of the hormone (119). Infusion of a recombinant adenovirus containing the mouse leptin cDNA into ob/ob mice reduced body weight and food intake relative to control ob/ob animals, and it normalized insulin levels and glucose tolerance (72). In these studies, leptin delivery was only sustained for a period of 2 weeks, after which leptin levels declined and food intake, body weight, and insulin resistance increased. More recently, AAV has been used instead of adenovirus to express leptin in ob/ob mice. This was accomplished by intramuscular injection of a recombinant AAV vector containing the cDNA encoding mouse leptin under control of the cytomegalovirus promoter (71). Injection of 5×10^{10}–1×10^{11} AAV particles resulted in a clear increase in circulating leptin and was accompanied by reduced food intake, a decline in body

weight, and normalization of blood glucose and insulin resistance. Remarkably, most of these measurements were made 6–8 weeks after vector injection, which suggests a considerably longer duration of expression than was achieved with the adenovirus vector in the ob/ob model.

The differing periods of transgene expression in the foregoing experiments are unexplained. One interesting observation is the relatively long duration of adenovirus-mediated delivery of leptin in normal rats (at least 28 days) compared with the shorter duration in the ob/ob mouse. Assuming that immunological responses to vector-encoded genes were similar in these two sets of experiments, the difference in duration between them could be related to recognition of the recombinant leptin as a novel antigen in the case of the ob/ob mouse studies, whereas tolerance to leptin may exist in the case of normal rats that are rendered hyperleptinemic. However, this would not explain the longer duration of expression of the AAV-delivered leptin in the ob/ob model, unless the AAV vector was much less antigenic than the adenovirus vector used in these animals, or the inflammatory response mounted against genetically altered muscle cells is milder compared with that against liver cells. Further investigation will be required to discriminate between these possibilities. The issue of whether the AAV vector actually integrated into muscle genomic DNA was also left uninvestigated and will be an important piece of information in comparing the utility of the two methods for future long-term peptide delivery studies. Nevertheless, these techniques provide powerful tools for delivery of hormones in whole animals, particularly for those peptides that require minimal proteolytic processing (there is no prohormone precusor form of leptin) or acute regulation of secretion.

CONCLUSIONS AND FUTURE DIRECTIONS

In this chapter, we reviewed recent applications of viral gene transfer technologies to metabolic and nutritional research. We focused on recombinant adenovirus as the vehicle of choice on the basis of (*a*) the ease of construction of these viruses, (*b*) the ability to produce high-titer stocks without the requirement for helper virus or sophisticated packaging cell lines, (*c*) the very high efficiency of gene transfer to relevant primary cell types such as hepatocytes, islets of Langerhans, and myocytes, and (*d*) the ability to use these vectors for efficient gene delivery to the liver of intact animals, an important organ of fuel homeostasis. These technologies allow us to manipulate specific steps in metabolic pathways to determine their importance in control of fuel metabolism. They also allow us to evaluate the function of new or disease-related genes in both in vitro and in vivo settings. Finally, recombinant adenovirus and AAV allow chronic delivery of peptide hormones in whole animals, enabling assessment

of long-term metabolic impact. All these applications may translate, in the future, into development of novel pharmacologic or gene therapeutic strategies for treatment of metabolic diseases. Ultimate success in application of these methods to humans will require further development of nonimmunogenic viral vectors that provide assurance of long-term transgene expression. Based on recent progress, these appear to be attainable goals, such that there is reason for cautious optimism that new strategies for treatment of metabolic diseases will emerge over the next decade.

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