Insulin-degrading enzyme identified as a candidate diabetes susceptibility gene in GK rats

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Genetic analysis of the diabetic GK rat has revealed several diabetes susceptibility loci. Congenic strains have been established for the major diabetes locus, Niddm1, by transfer of GK alleles onto the genome of the normoglycemic F344 rat. Niddm1 was dissected into two subloci, physically separated in the congenic strains Niddm1b and Niddm1i, each with at least one disease susceptibility gene. Here we have mapped Niddm1b to 1 cM by genetic and pathophysiological characterization of new congenic substrains for the locus. The gene encoding insulin-degrading enzyme (Ide) was located to this 1 cM region, and the two amino acid substitutions (H18R and A890V) identified in the GK allele reduced insulin-degrading activity by 31% in transfected cells. However, when the H18R and A890V variants were studied separately, no effects were observed, demonstrating a synergistic effect of the two variants on insulin degradation. No effect on insulin degradation was observed in cell lysates, indicating that the effect is coupled to receptor-mediated internalization of insulin. Congenic rats with the Ide GK allele displayed post-prandial hyperglycemia, reduced lipogenesis in fat cells, blunted insulin-stimulated glucose transmembrane uptake and reduced insulin degradation in isolated muscle. Analysis of additional rat strains demonstrated that the dysfunctional Ide allele was unique to GK. These data point to an important role for Ide in the diabetic phenotype in GK.

INTRODUCTION

Type 2 diabetes, or non-insulin-dependent diabetes mellitus (NIDDM), is an accelerating health burden in urbanized societies with aging populations. The disease is associated with physical inactivity, dyslipidemia, obesity and other components of the metabolic syndrome. A continuously increasing number of people worldwide are affected and are therefore at risk for myocardial infarction, stroke, end-stage kidney disease, vision defects and neurological problems.

It is generally considered that the disease in most cases results from a combination of impaired insulin action in target tissues and a reduced capacity to secrete insulin from the pancreatic B cells. The etiology is multifactorial with an appreciable genetic basis, although environmental factors are also of critical importance for the development of overt disease (1–6). In the vast majority of type 2 diabetes patients, the genetic basis and the pathophysiological mechanisms behind the disease are still unclear. Genetic dissection of type 2 diabetes in humans is compromised by genetic heterogeneity, multigenicity and environmental variation. However, mutations in several genes linked to monogenic forms of type 2 diabetes have been identified in recent years (7–12).

One way to circumvent the complexity in the human population is to genetically dissect well-defined animal models of multifactorial diseases. Such dissections of rat and mouse models for the metabolic syndrome have successfully revealed loci for glucose intolerance and diabetes (13–20). The inbred GK rat is an extensively studied model of type 2 diabetes that displays the key features of the disease, such as defects in both insulin action and secretion as well as late complications (21,22). The genetic analysis of the GK rat revealed several loci for diabetes-associated phenotypes (13,14). We identified three genome-wide significant quantitative trait loci (QTL) (Niddm1, Niddm2 and Niddm3) affecting glucose homeostasis, one locus controlling body weight (Weight1) and an additional 10 loci with less significant influences on diabetes-associated phenotypes (13). The major QTL, Niddm1, located on chromosome 1, explained 31% of the genetic variation of post-prandial glycemia and influenced insulin levels and body weight. The Niddm1 locus has been dissected further by analysis of congenic strains established by transferring Niddm1 GK alleles onto the genome of the normoglycemic F344 rat (23). This made possible the dissection of Niddm1 into two genetic entities defined by the two non-overlapping congenic strains Niddm1b and Niddm1i, each displaying a distinct diabetic phenotype.

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Here we describe the submapping of the Niddm1b locus to a small genetic region of 1 cM defined by new congenic strains. The gene encoding insulin-degrading enzyme (Ide) was mapped within this region, and a GK-specific allelic variant of Ide was identified. Furthermore, in vitro expression analysis showed reduced insulin degradation by the GK variant, and isolated muscle from a congenic strain with the Ide GK allele demonstrated decreased insulin degradation and blunted insulin-stimulated glucose transport.

**RESULTS**

**Analysis of diabetes-related phenotypes in Niddm1b substrains**

Substrains of Niddm1b were established as described in Materials and Methods. Since an intraperitoneal glucose tolerance test (IPGTT) was used to identify Niddm1 as well as to define the Niddm1b and Niddm1i subloci, the same test was applied to characterize the new congenic sublines for Niddm1b (Fig. 1). To map the susceptibility gene within Niddm1b, male rats from the congenic strains (Niddm1e, Niddm1f and Niddm1c) were subjected to an IPGTT at 95 days of age with F344 as control. Similarly to Niddm1b (23), post-prandial glucose levels in both Niddm1e and Niddm1f were significantly higher compared with F344 (Table 1). The most pronounced difference was observed at 30 min after glucose injection, when the glucose levels were 21% higher in both Niddm1e and Niddm1f. The basal and 30 min insulin levels were also significantly higher than in F344. The glucose and insulin values in Niddm1c were almost indistinguishable from those in F344, and therefore the differences between Niddm1c and Niddm1e or Niddm1f will be the same as between F344 and Niddm1e or Niddm1f.

Following the IPGTT at 95 days, the animals were treated with a high fat diet to challenge the metabolism further. The congenic strains exhibiting a diabetic phenotype at 95 days (Niddm1e and Niddm1f) and F344 control rats were treated with high fat diet from age 120 days and were subjected to an IPGTT at 225 days of age. Subsequently, basal levels of triglycerides, total cholesterol, high density lipoprotein (HDL) cholesterol and low density lipoprotein (LDL) cholesterol were determined, and the epididymal fat depots were weighed. At 225 days of age, the post-prandial glucose levels were again significantly higher in Niddm1e and Niddm1f compared with

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**Table 1. Diabetes-associated phenotypes in Niddm1c, Niddm1e, Niddm1f and F344 at age 95 days**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>F344 (n = 12)</th>
<th>Niddm1e (n = 10)</th>
<th>Niddm1f (n = 11)</th>
<th>Niddm1c (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>268 ± 4</td>
<td>276 ± 3</td>
<td>277 ± 5</td>
<td>258 ± 5</td>
</tr>
<tr>
<td>Glucose 0 min</td>
<td>5.3 ± 0.1</td>
<td>5.4 ± 0.1</td>
<td>5.7 ± 0.1</td>
<td>5.6 ± 0.2</td>
</tr>
<tr>
<td>Glucose 15 min</td>
<td>15.4 ± 0.4</td>
<td>17.2 ± 0.7*</td>
<td>17.3 ± 0.7</td>
<td>15.6 ± 0.6</td>
</tr>
<tr>
<td>Glucose 30 min</td>
<td>12.0 ± 0.3</td>
<td>14.5 ± 0.3***</td>
<td>14.5 ± 0.5***</td>
<td>11.8 ± 0.5</td>
</tr>
<tr>
<td>Glucose 60 min</td>
<td>6.8 ± 0.1</td>
<td>8.4 ± 0.3***</td>
<td>7.7 ± 0.2***</td>
<td>6.1 ± 0.2*</td>
</tr>
<tr>
<td>Glucose 90 min</td>
<td>6.3 ± 0.1</td>
<td>6.7 ± 0.1</td>
<td>6.8 ± 0.3</td>
<td>6.6 ± 0.2</td>
</tr>
<tr>
<td>Insulin 0 min</td>
<td>201 ± 25</td>
<td>331 ± 24**</td>
<td>441 ± 37***</td>
<td>246 ± 57</td>
</tr>
<tr>
<td>Insulin 15 min</td>
<td>2162 ± 167</td>
<td>2425 ± 102</td>
<td>2431 ± 215</td>
<td>2265 ± 373</td>
</tr>
<tr>
<td>Insulin 30 min</td>
<td>1626 ± 164</td>
<td>2497 ± 93***</td>
<td>2405 ± 162**</td>
<td>1554 ± 320</td>
</tr>
</tbody>
</table>

All values are given as means ± SEM. Glucose values are given in mmol/l and insulin in pmol/l. Each congenic strain was compared with F344 (Student’s t-test), and significant differences are indicated: *P < 0.05; **P < 0.01; ***P < 0.001.
DNA sequence and expression analysis of Ide

Candidate genes were identified by employing genetic mapping data for human and mouse. Synteny is conserved between the Niddm1 region on rat chromosome 1, human chromosomes 9 and 10 and mouse chromosome 19 (23). The most interesting candidate was the gene encoding insulin-degrading enzyme (IDE), mapped to chromosome 10q24 in the human and chromosome 19 in the mouse. The Ide gene was genetically mapped on rat chromosome 1 within the GK interval of Niddm1e (Fig. 1).

To investigate the hypothesis that changes in the IDE protein could explain the phenotype of Niddm1e, the cDNA sequence of IDE was determined in both GK and F344. Sequencing the complete translated region of the gene revealed three nucleotide differences between GK and F344 in the coding region, one in the 5′ end (codon 18) and two in the 3′ end (codons 890 and 934). Two of these resulted in amino acid substitutions, a silent 934 codon variation in 3 of the 11 strains (Table 3).

By studying the effect of the GK variant of IDE, the insulin-degrading activity was determined in an in vitro expression system. IDE was overexpressed in transfected COS-1 cells, and the ability of living and homogenized cells to hydrolyze insulin was recorded. Western blot analysis with anti-IDE anti-

### Table 2. Diabetes-associated phenotypes in Niddm1e, Niddm1f and F344 at age 225 days

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>F344 (n = 12)</th>
<th>Niddm1e (n = 10)</th>
<th>Niddm1f (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>377 ± 6</td>
<td>400 ± 5*</td>
<td>384 ± 8</td>
</tr>
<tr>
<td>Glucose 0 min</td>
<td>4.8 ± 0.1</td>
<td>4.9 ± 0.1</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td>Glucose 15 min</td>
<td>17.1 ± 0.3</td>
<td>20.3 ± 1.3*</td>
<td>17.1 ± 1.4</td>
</tr>
<tr>
<td>Glucose 30 min</td>
<td>17.7 ± 0.8</td>
<td>19.4 ± 0.6</td>
<td>18.5 ± 0.7</td>
</tr>
<tr>
<td>Glucose 60 min</td>
<td>14.6 ± 0.7</td>
<td>17.6 ± 1.0*</td>
<td>17.8 ± 0.6**</td>
</tr>
<tr>
<td>Glucose 90 min</td>
<td>10.4 ± 0.4</td>
<td>13.9 ± 0.9***</td>
<td>13.4 ± 0.8**</td>
</tr>
<tr>
<td>Insulin 0 min</td>
<td>337 ± 16</td>
<td>480 ± 46**</td>
<td>410 ± 24*</td>
</tr>
<tr>
<td>Insulin 15 min</td>
<td>1069 ± 109</td>
<td>1166 ± 143</td>
<td>985 ± 111</td>
</tr>
<tr>
<td>Insulin 30 min</td>
<td>1217 ± 77</td>
<td>1197 ± 144</td>
<td>1049 ± 71</td>
</tr>
<tr>
<td>Fat weight (g)</td>
<td>8.5 ± 0.3</td>
<td>10.6 ± 0.5***</td>
<td>9.3 ± 0.4</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>0.66 ± 0.03</td>
<td>0.76 ± 0.06</td>
<td>0.61 ± 0.02</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>4.35 ± 0.13</td>
<td>4.33 ± 0.20</td>
<td>4.13 ± 0.15</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>1.23 ± 0.07</td>
<td>0.97 ± 0.02**</td>
<td>1.12 ± 0.03</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>2.80 ± 0.08</td>
<td>3.00 ± 0.17</td>
<td>2.77 ± 0.16</td>
</tr>
</tbody>
</table>

All values are given as means ± SEM. Glucose, cholesterol and triglyceride values are given in mmol/l and insulin in pmol/l. Each congenic strain was compared with F344 (Student’s t-test), and significant differences are indicated: *P < 0.05; **P < 0.01; ***P < 0.001.
bodies confirmed expression of transfected IDE cDNAs (data not shown). The two amino acid variants, H18R and A890V, in the GK allele were studied separately as well as in combination, and compared with the expressed F344 allele. The insulin-degrading activity in intact cells transfected with the GK allele, containing both H18R and A890V, was decreased by 31% (P = 0.002) compared with F344 (Fig. 3). However, when the two variants were analyzed separately, no significant decrease in activity of either variant was observed. Thus, the data indicate a synergistic effect of the two amino acid substitutions. No allele-specific differences were found in enzyme activity analyzed in cell lysates. We conclude that *Ide* is a likely candidate for the diabetes-associated phenotypes in Niddm1e.

**Fine mapping of the diabetes-associated phenotype**

To support further the notion that IDE affects the diabetes phenotype in GK, a new congenic strain (Niddm1g) was established and characterized (Fig. 1). Niddm1g covered 17 cM of GK alleles and overlapped both Niddm1e and Niddm1f except for ~1 cM in which *Ide* was located. Thus, Niddm1g was predicted to contain the F344 alleles of *Ide*, which was confirmed by sequencing. Niddm1g together with Niddm1e and F344 as controls were subjected to an IPGTT. The glucose levels of Niddm1e were higher than those of Niddm1g at 60 min (P = 0.03) and 90 min (P = 0.02). Results are shown as means ± SEM.

### Table 3. Sequence variants in the *Ide* gene of various rat strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Codon 18</th>
<th>Codon 890</th>
<th>Codon 934</th>
<th>Holder</th>
</tr>
</thead>
<tbody>
<tr>
<td>GK</td>
<td>CGC (Arg)</td>
<td>GTG (Val)</td>
<td>GAC</td>
<td>Karolinska Institutet, Stockholm, Sweden</td>
</tr>
<tr>
<td>FRL</td>
<td>CGC (Arg)</td>
<td>GCG (Ala)</td>
<td>GAC</td>
<td>Flinders University, Flinders, Australia</td>
</tr>
<tr>
<td>FSL</td>
<td>CGC (Arg)</td>
<td>GCG (Ala)</td>
<td>GAC</td>
<td>Flinders University, Flinders, Australia</td>
</tr>
<tr>
<td>Bkl:SD</td>
<td>CGC (Arg)</td>
<td>GCG (Ala)</td>
<td>GAC</td>
<td>B &amp; K Universal AB, Sollentuna, Sweden</td>
</tr>
<tr>
<td>ACI/SegHsd</td>
<td>CGC (Arg)</td>
<td>GCG (Ala)</td>
<td>GAT</td>
<td>Harlan, Indianapolis, IN</td>
</tr>
<tr>
<td>DA/Han</td>
<td>CGC (Arg)</td>
<td>GCG (Ala)</td>
<td>GAT</td>
<td>Central Institute for Laboratory Animal Breeding, Hannover, Germany</td>
</tr>
<tr>
<td>BB</td>
<td>CAC (His)</td>
<td>GCG (Ala)</td>
<td>GAT</td>
<td>Karolinska Institutet, Stockholm, Sweden</td>
</tr>
<tr>
<td>BN/SnOlaHsd</td>
<td>CAC (His)</td>
<td>GCG (Ala)</td>
<td>GAT</td>
<td>Harlan, Blackthorn, UK</td>
</tr>
<tr>
<td>COP/Hsd</td>
<td>CAC (His)</td>
<td>GCG (Ala)</td>
<td>GAT</td>
<td>Harlan, Indianapolis, IN</td>
</tr>
<tr>
<td>F344/CrlBR</td>
<td>CAC (His)</td>
<td>GCG (Ala)</td>
<td>GAT</td>
<td>Charles River Laboratories, Wilmington, MA</td>
</tr>
<tr>
<td>LEW/CrlBR</td>
<td>CAC (His)</td>
<td>GCG (Ala)</td>
<td>GAT</td>
<td>Charles River Laboratories, Wilmington, MA</td>
</tr>
<tr>
<td>PVG/Bkl</td>
<td>CAC (His)</td>
<td>GCG (Ala)</td>
<td>GAT</td>
<td>B &amp; K Universal AB, Sollentuna, Sweden</td>
</tr>
<tr>
<td>Bkl:WIST</td>
<td>CAC (His)</td>
<td>GCG (Ala)</td>
<td>GAT</td>
<td>B &amp; K Universal AB, Sollentuna, Sweden</td>
</tr>
</tbody>
</table>

**Figure 3.** Enzyme activities of IDE variants in transfected intact COS-1 cells. Activities are from COS-1 cells transfected with *Ide* alleles from GK, F344 and the individual GK variants, A890V and H18R. All values are from four separate transfections and are expressed as a percentage of F344 activity, which was defined arbitrarily as 100%. Within each experiment, the background COS-1 insulin-degrading activity was subtracted from each individual value, and activities were corrected for the total protein content. Significant differences compared with the F344-allele are indicated: **P = 0.002. The actual values (means ± SEM) for A890V, H18R and GK are 95 ± 9, 89 ± 8 and 69 ± 6%, respectively.

**Figure 4.** Pathophysiological characterization of the Niddm1g congenic strain. Male rats (140 days) from strains Niddm1e (n = 7), Niddm1g (n = 8) and F344 (n = 8) were subjected to IPGTT, and blood glucose levels were determined at the indicated time points. The glucose levels were higher in Niddm1e than in F344 at 15 min (P = 0.01), 60 min (P = 0.01) and 90 min (P = 0.002). As compared with Niddm1g, the glucose levels in Niddm1e were higher at 60 min (P = 0.03) and 90 min (P = 0.02). Results are shown as means ± SEM.
Glucose transport and insulin degradation in muscle. Insulin-stimulated 3-O-methylglucose transport in isolated EDL muscle and insulin-degrading activities in isolated epitrochlearis muscles of male F344 (n = 6), Niddm1e (n = 6) and Niddm1g (n = 6) rats. (a) Insulin-stimulated glucose transport is expressed as fold increase over basal. The increase in glucose transport was significantly lower in muscle from Niddm1e at an insulin concentration of 0.6 nM (P = 0.04) in comparison with F344. As compared with Niddm1g, the increase in muscle from Niddm1e was lower at 0.6 nM (P = 0.005) and 2.4 nM (P = 0.04) insulin. Results are shown as means ± SEM. (b) Insulin-degrading activity is expressed as a percentage of F344 activity, arbitrarily defined as 100%. Activities were corrected for differences in muscle weight. Significant difference compared with F344 are indicated: **P = 0.007. The values for IDE activity in muscle from Niddm1e and Niddm1g are 79 ± 4 and 91 ± 3% (means ± SEM), respectively.

The effect of the GK allele in the 1 cM region that differs between Niddm1e and Niddm1g.

The phenotype of Niddm1e indicated that the primary defect is in insulin action rather than in insulin secretion. In addition to the phenotype of Niddm1e, we mapped the GK haplotype in the 1 cM region that differs between F344 and Niddm1g for either basal or insulin-induced glucose transport.

In isolated muscle from these strains, the insulin-degrading activity was also determined. The epitrochlearis muscles were isolated from Niddm1e, Niddm1g and F344, and the abilities of the muscles to degrade insulin were determined. The insulin-degrading activity in muscle from Niddm1e was significantly reduced by 21% (P = 0.007) compared with F344 (Fig. 5b). No significant difference was observed between F344 and Niddm1g.

**DISCUSSION**

We describe the establishment of congenic substrains for the diabetes susceptibility locus Niddm1b (23), and show that the diabetes phenotype is retained in the new congenic strain Niddm1e that only maintains 3.7 cM of the 28 cM GK region of Niddm1b. Similarly to the parental Niddm1b strain, Niddm1e displayed elevated post-prandial glucose levels, impaired basal and insulin-induced lipogenesis in isolated adipocytes, increased body weight, excess epididymal fat mass and elevated fasting and post-prandial insulin concentrations (Tables 1 and 2). To challenge further the metabolism of the animals and to investigate the effects of high fat diet and age on the development of the diabetic phenotype, old rats (225 days) were subjected to an IPGTT after 3.5 months on a high fat diet (Table 2). At this age, F344 control rats also showed signs of impaired glucose tolerance. The post-prandial glucose levels in F344 were distinctly higher than at 95 days, and glucose levels were not normalized 90 min after glucose injection, as at 95 days. This effect was even more pronounced in the congenic strains (Niddm1e and Niddm1f) that exhibited the most significant difference compared with F344 at the 90 min time point. We conclude that increasing age and high fat diet significantly aggravated the diabetes-associated phenotypes in Niddm1e compared with F344, demonstrating that Niddm1e carries a diabetes susceptibility gene. As in the parental Niddm1b strain, the phenotype of Niddm1e indicated that the primary defect is in insulin action rather than in insulin secretion. In addition to high blood glucose levels, Niddm1e rats showed both reduced glucose incorporation into lipids in isolated adipocytes (Fig. 2) and blunted insulin-induced glucose transport in muscle (Fig. 5a). Also, the basal in vivo hyperinsulinemia at 95 and 225 days indicated insulin resistance (Tables 1 and 2). At 95 days of age, the insulin levels at 30 min were elevated in Niddm1e compared with F344, indicating a compensatory insulin secretion at the prevailing insulin resistance. At 225 days, the insulin levels after glucose injection in Niddm1e were not different compared with F344, despite increased glucose levels, suggesting that insulin secretion defects had developed, probably secondarily to insulin resistance. We thus conclude that the Niddm1b diabetes susceptibility locus is redefined to the 3.7 cM GK region remaining in the Niddm1e strain.

In the GK-specific 3.7 cM region of Niddm1e, we mapped the Ide gene, an interesting candidate for the diabetes phenotype. Ide is a Zn2+-requiring metalloproteinase with a distinct Zn2+-binding site (24,25), and it is widely expressed throughout the body (26). Several studies support Ide as part of the primary enzymatic mechanism for initiating cellular insulin processing and degradation; it is likely that Ide has an important regulatory role in insulin action (27). On binding to...
its receptor, insulin is internalized into endosomes where insulin degradation is initiated by IDE (28). After dissociation, the insulin receptor is recycled to the plasma membrane. Apart from endosomes, IDE is also present in the cell membrane, in the cytoplasm and in peroxisomes. Several additional peptides interact with IDE, e.g. glucagon, ANP, transforming growth factor-α and insulin-like growth factor (IGF) II bind and are readily degraded. In addition, proinsulin, IGF-I and epidermal growth factor bind, but are poorly degraded by IDE (27). IDE may also be involved in other types of cellular proteolysis and interacts with the proteasome, the major site for intracellular protein degradation (29). Furthermore, IDE may have a regulatory role in steroid action and interacts with the receptors for glucocorticoid and androgen (30).

The Ide cDNA was sequenced in GK and F344, in search of structural differences in the encoded protein that could mediate the diabetic phenotype in Niddm1e. Three single nucleotide polymorphisms were identified; two of these resulted in amino acid substitutions (H18R and A890V), whereas the third was silent (codon 934). The Ide cDNA was sequenced in 11 additional rat strains to investigate the prevalence of the identified variants. A890V was unique for GK, whereas H18R and the variant at codon 934 were also present in other strains, supporting the argument that the A890V variant influences the diabetic phenotype in Niddm1e (Table 3). Furthermore, A890 is conserved in Ide from rat, human and Drosophila, suggesting that this residue is important for enzymatic activity. The substitutions are not in residues that have been shown to be necessary for Zn binding or for catalytic activity (31). However, the residues important for ligand binding are still unknown.

In vitro expression of IDE revealed that the GK allele had a 31% reduced ability to degrade insulin in living cells. However, when the H18R and A890V variants were studied separately, no significant effects were observed (Fig. 3). This indicates that the two variants are interacting (synergism) to mediate the effect on insulin degradation. No effects of the GK variants on insulin degradation were observed in cell lysates of Ide-transfected COS cells, suggesting that the defect in IDE is coupled to receptor-mediated internalization of insulin or other activities that require intact cell structures.

These data suggest that Ide is one of the genes contributing to the diabetes phenotype in GK rats and mediates the hyperglycemia and insulin resistance observed in Niddm1e, possibly through an impaired insulin-degrading capacity. This is supported by both genetic and pathophysiological data: (i) Niddm1g overlaps the major part of the GK region in Niddm1e except for 1 cM that includes Ide; (ii) the normoglycemic Niddm1g strain displayed post-prandial glucose levels indistinguishable from those observed in the F344 control rat (Fig. 4); and (iii) the insulin-induced glucose transport is severely reduced in muscle from Niddm1e together with a 21% reduced ability to degrade insulin (Fig. 5). These phenotypes were not observed in the congenic strain Niddm1g, which carries F344 alleles of Ide. Thus, the diabetes susceptibility locus is restricted to the 1 cM region that encompasses Ide, and which encodes several phenotypes related to IDE action, most conspicuously the reduced insulin-stimulated transmembrane transport of glucose in muscle. In this region of the rat genome, 1 cM in the (GK × F344) genetic map corresponds to only 0.5 Mb of DNA, as deduced by comparison with the radiation hybrid map (32).

Several studies of diabetes in humans have shown decreased insulin clearance and degradation associated with insulin resistance. This was observed in obese hyperinsulinemic individuals and in type 2 diabetes patients (33–36), in non-diabetic insulin-resistant individuals (37) and in two different ethnic groups at high risk for diabetes (38,39). In contrast, other studies have demonstrated an increased insulin clearance and degradation associated with diabetes (40,41). However, increased insulin degradation seems to be associated with insulin deficiency as such and, in both type 2 diabetes patients and in streptozotocin diabetic rats, increased degradation can be restored by insulin treatment (41,42). Increased insulin degradation in target tissues could therefore also be a secondary event in diabetes to compensate for impaired insulin secretion.

Furthermore, genetic linkage has been reported between type 2 diabetes and the distal region of chromosome 10q, close to the Ide gene in humans. In a Mexican American population, evidence was found for a susceptibility locus on 10q that influenced both type 2 diabetes and age at onset of disease (43). Weak linkage to type 2 diabetes was also observed in approximately the same region among Utah Caucasians (44). Moreover, a locus with suggestive evidence of linkage to leptin levels was found on 10q in French families (45).

Studies on the mechanisms of IDE that ultimately may lead to diabetes are in an early phase. Presently, we can only speculate about the biochemical mechanisms through which IDE could mediate the insulin resistance phenotype observed in vivo in Niddm1e rats, in fat cells as well as the blunted insulin-stimulated glucose transport in muscle from Niddm1e. However, previous studies of rats and humans have implied that both insulin receptor internalization and receptor recycling defects are associated with insulin resistance and diabetes (36,46,47). Possibly, a decreased intracellular degradation of insulin bound to its receptor would inhibit receptor-mediated signal transduction, by lowering the number of available receptors on the cell membrane and/or compromising the downstream signaling from the receptor. In addition to an effect on insulin receptor recycling and signaling, reduced IDE activity may also have a diabetogenic effect through its ability to degrade peptides sharing a hydrophobic motif (48), that has similarities to a motif common to amyloid-forming peptides (49). This notion may also be important for the susceptibility to diabetes and its complications, i.e. those affecting pancreas, large and small blood vessels and the basal membrane of the glomeruli of the kidney.

**MATERIALS AND METHODS**

**Rat breeding**

Inbred Fischer-344 rats were purchased from Charles River Laboratories (Wilmington, MA) and maintained by brother-sister mating. Rats had free access to tap water and chow and were maintained on a 12 h light and dark cycle (6:00–18:00 h). The high fat diet contained 2% cholesterol, 20% olive oil and 0.5% bile acid mixed in standard chow. Rats were fed the high fat diet from the age of 120 days to killing. The congenic strains were bred by transferring the GK-derived genetic
interval onto the F344 strain (23). The new sublines from Niddm1b (23) were established by backcrossing of Niddm1b rats to F344, and recombinants were identified within the Niddm1b region. The Niddm1e, Niddm1f, Niddm1c and Niddm1g congenic strains were bred by 12 successive backcrosses to F344 followed by intercrosses to establish homozygous lines.

**IPGTT and lipid analysis**

The IPGTTs were performed in male rats, as previously described (23). The serum insulin levels were determined with a rat-specific enzyme-linked immunosorbent assay (ELISA; Mercodia AB, Uppsala, Sweden). The insulin values in micrograms per liter obtained from the ELISA analysis were converted to picomoles per liter by multiplying by a factor of 174. The serum levels of triglycerides, total cholesterol and HDL cholesterol were determined with Vitros TRIG Slides, Vitros CHOL Slides (Johnson & Johnson Clinical Diagnostics, Rochester, NY) and Liquid N-geneous HDL-c reagent kit (Biomed-RK, Jönköping, Sweden).

**Analysis of lipogenesis**

Male rats (75 days) were decapitated after carbon dioxide anesthesia, and the epididymal fat depots (1–2 g) were removed. Adipocytes were prepared as described (50). Studies of glucose incorporation into lipids (lipogenesis) were performed at a glucose concentration of 1 µM, at which glucose transport into the cells is rate limiting (51). The adipocytes were incubated at 2% (v/v) concentration in 0.5 ml of Krebs–Ringer phosphate buffer containing 40 mg/ml albumin (Sigma, Stockholm, Sweden), 0.2 µM [3-3H]glucose (5 x 10⁶ c.p.m.), 1.0 µM unlabeled glucose and increasing concentrations of insulin. The analysis was performed in triplicate at each insulin concentration at 37°C for 2 h. Reactions were terminated by rapid chilling to 4°C. Incorporation of glucose into lipids was determined as described (52). Briefly, 45 µl of 6.0 M H₂SO₄ and 4.0 ml of toluene with 2,5-diphenyl-p-oxazole were added to each vial, and the vials were left at room temperature overnight before liquid scintillation counting. Lipogenesis was expressed per cell surface area in order to eliminate differences depending solely on adipocyte size (53). The maximal insulin-induced lipogenesis was calculated as the difference between glucose incorporation at maximum minus the incorporation in the absence of insulin.

**Genotype analysis and localization of markers**

Rats were genotyped by PCR amplification of microsatellite markers essentially as previously described (54), with the exception that [γ-³²P]ATP was used to label one of the primers in each pair. For the genetic mapping of new markers, the 45 rats with the most extreme glucose values from our first F₂ cross were genotyped (13), and markers were placed on the genetic map by employing the computer package Mapmaker/exp 3.0 (55).

**Genetic mapping of Ide**

The Ide probe for hybridization was synthesised by RT–PCR, using available rat cDNA sequences and gene-specific primers (56). For the reverse transcriptase reaction, total RNA was prepared from the whole body of a 1-day-old rat, as previously described (57). A 6 µg aliquot of RNA was transcribed in a total volume of 20 µl using BRL Superscript II (Life Technologies, Täby, Sweden), as described by the manufacturer. Ide mRNA was reverse transcribed with primer 5'-AGCTGGTG-GGACAAACAGGAG-3' and 2 µl of the reverse transcriptase reaction was introduced in the subsequent PCR amplification (primers: 5'-GTGAACCTGCTGTAACTAAG-3' and 5'-AGCTGGTGACAAACAGGAG-3'; PCR-profile: 94°C for 4 min, 30 cycles consisting of 94°C for 30 s, 55°C for 1 min, 72°C for 2 min, and a final extension for 7 min at 72°C). The Southern blots analysis was performed as described (58). High molecular weight DNA was extracted from rat liver, and genomic DNA (10 µg) was digested with the restriction endonuclease, size fractionated in 0.8% agarose gels and transferred to nylon membranes (Zeta-probe; Bio-Rad, Hercules, CA). The restriction fragment length polymorphism (RFLP) probes were ³²P-labeled by random priming (59). An HincII RFLP was identified that generated fragments of 2.7 kb in GK and 0.7 kb in F344.

**Sequencing of rat IDE cDNA**

A 3128 bp rat Ide cDNA fragment, amplified by RT–PCR with gene-specific primers, was sequenced. A 6 µg aliquot of total RNA prepared from rat liver was used in the 20 µl reverse transcriptase reaction with cDNA primer 5'-CTGTTTG-TGCTCTCTTAAATTGC-3'. A 2 µl aliquot of the reverse transcriptase reaction was introduced in the PCR, using Expand Long Template PCR System (Boehringer Mannheim, Mannheim, Germany) as described by the manufacturer (PCR primers: 5'-ATGCCGAACGGGCTCGTGTG-3' and 5'-AGCCGAAAACCTACTCAAACG-3'; PCR profile: 94°C for 2 min, 30 cycles consisting of 94°C for 10 s, 54°C for 30 s, 68°C for 2.5 min, of which the last 20 cycles were elongated for 20 s at 68°C for each cycle, and a final extension for 7 min at 68°C). The DNA sequences of the RT–PCR products were determined using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and Ide-specific primers in an ABI PRISM 377 semi-automatic sequencer (Applied Biosystems, Foster City, CA).

**Plasmid construction and COS-1 cell transfections**

Ide mRNA from GK and F344 was amplified by RT–PCR essentially as described above with primers extended with restriction sites. The resulting 3.1 kb cDNA product containing the complete translated region was ligated into the BgIII and MluI restriction sites of the expression vector pCMV4 (D.W. Russel, University of Texas Southwestern Medical Center, Houston, TX) under control of the strong cytomegalovirus promoter. The Ide cDNA inserts in the resulting constructs pCMV4-Ide(GK) and pCMV4-Ide(F344) were sequenced to exclude PCR artifacts. Internal restriction sites were used to separate the GK sequence variants generating pCMV4-Ide(H18R) and pCMV4-Ide(A890V). Approximately 6 x 10⁶ COS-1 cells were transiently transfected by electroporation (Gene Pulser; Bio-Rad, Richmond, CA; 1200 V, 25 µF) with 10 µg of pCMV4-Ide plasmid together with 1 µg of the β-galactosidase vector pCH110 (Pharmacia, Upppsala, Sweden).
Assay of insulin-degrading activity in COS-1 cells

Transfected COS-1 cells were seeded in 6 cm Petri dishes and incubated for 36 h in Dulbecco’s modified Eagle’s medium (DMEM) supplied with 10% calf serum. Subsequently, the cells were washed twice in phosphate-buffered saline (PBS), pre-incubated at 37°C in 3 ml of DMEM supplemented with 1 mg/ml bovine serum albumin (BSA) for 30 min, and finally incubated in 2 ml of DMEM containing 1 mg/ml BSA and 15 000 c.p.m./ml of [125I]insulin. Triplicate aliquots of 100 µl were removed at 30, 45 and 60 min after addition of insulin. Undegraded insulin was precipitated for 30 min on ice with 1 vol of 25% trichloroacetic acid (TCA). The samples were centrifuged at 20 000 g for 10 min, the supernatant recovered, and the amount of degraded insulin measured by radioactive counting. Cells were washed twice further with PBS, incubated for 2 h in DMEM, trypsinized and washed three times in PBS. The cells (~3 x 10⁶ cells per plate) were recovered for homogenization by sonication for 15 s in 300 µl of 100 mM phosphate buffer (pH 7.4) containing 0.5 mg/ml BSA. The homogenate was centrifuged at 350 g for 10 min, and the supernatant was collected for measurement of insulin degradation activity, protein concentration, β-galactosidase activity (verifying the reproducibility of transformation efficiency) and western blot analysis. Triplicate aliquots of cell lysates containing 1 µg of protein were incubated for 15 min at 37°C in 100 µl of assay buffer containing 2000 c.p.m. of [125I]insulin, and the amount of degraded insulin was measured as described above. In all experiments, background COS-1 insulin-degrading activity (in cells transfected with pCMV plasmid) was 20–25% of that in cells expressing F344 rat IDE. The IDE protein was detected by immunoblotting according to standard procedures using IDE antibodies kindly provided by Dr M.R. Rosner (University of Chicago, Chicago, IL).

Muscle preparation

Male rats (3 months old) were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt). Isolated EDL and epitrochlearis muscles were incubated in vitro. Each EDL muscle was split into two equal longitudinal portions before incubation. Thus, four EDL muscle strips were prepared for each animal. The procedure and suitability of the muscle preparation have been described previously (60,61). All incubation media were prepared from a stock solution of pre-gassed (95% O₂/5% CO₂) Krebs–Henseleit buffer (KHB), which contained 5 mM HEPES and 0.1% BSA (radioimmunoassay grade). The gas phase in the vials was maintained at 95% O₂/5% CO₂ throughout all incubations.

Assessment of glucose transport activity in muscle

Glucose transport was assessed in EDL muscle using the glucose analog 3-O-methylglucose as described (60). EDL muscles were incubated at 30°C for 30 min in KHB supplemented with 5 mM glucose and 15 mM mannitol. Muscles were then incubated for an additional 30 min in KHB (5 mM glucose and 15 mM mannitol) with or without insulin (0.6 or 2.4 nM). Subsequently, muscles were incubated for 10 min in KHB (20 mM mannitol) with or without insulin as indicated above. Thereafter, muscles were incubated in KHB containing 8 mM 3-O-methylglucose (2.5 µCi/mmol) and 12 nM [14C]mannitol (26.3 µCi/mmol) for 12 min (insulin-stimulated) or 20 min (basal). Muscles were processed for glucose transport as described (60). Results are reported as insulin-stimulated fold increase of glucose analog accumulated per milliliter of intracellular water per hour over basal.

Assay of insulin-degrading activity in muscle

Determination of insulin degradation in intact muscle was performed essentially as previously described (40). Intact epitrochlearis muscles were washed in KHB (20 mM mannitol) at 37°C for 20 min, three times. Muscles were then incubated in 1 ml of KHB containing 10 000 c.p.m./ml of [125I]insulin for 30 min at 37°C. At the end of the incubation, triplicate 150 µl aliquots were removed and the amount of degraded insulin was measured by TCA precipitation (as described above).

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