a Brinkmann polytron and centrifuged at 17,700*g* for 45 min at 4°C. The pellet was resuspended in homogenization buffer (300 ml) and is referred to as the membrane fraction. The membrane fraction was incubated for 30 min at 4°C with an equal volume of 2× lysis buffer [1× lysis buffer consists of 10 m*M* tris-HCl (*p*H 7.5), 50 m*M* NaCl, 5 m*M* EDTA, 1% Triton X-100, 50 m*M* NaF, 150 μ*M* sodium orthovanadate, 30 m*M* sodium pyrophosphate, 1 m*M* PMSF, aprotinin (20 to 30 KIU/ml), leupeptin (10 μg/ml), and pepstatin (1 μg/ml)], and then centrifuged at 31,000*g* for 30 min. The supernatant was applied batchwise to a 150-ml WGA-Sepharose 4B column. The column was then washed with 300 ml of lysis buffer followed by 500 ml of column buffer, which contained 20 m*M* Hepes (*p*H 7.5), 2 m*M* EDTA, 10% glycerol, 0.1% Triton X-100, 50 m*M* NaF, 150 μ*M* sodium orthovanadate, 30 m*M* sodium pyrophosphate, 1 m*M* PMSF, aprotinin (20 to 30 KIU/ml), leupeptin (10 μg/ml), and pepstatin (1 μg/ml). Protein was eluted from the column with column buffer containing 0.5*M* *N*-acetylglucosamine. Peak protein containing fractions were combined and stored at −70°C.

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41. Supported by NIH grant R01 HL32898 (L.T.W.). V.A.F. is funded by CA 21765 from the National Cancer Institute and the American Lebanese Syrian Associated Charities. We thank J. Lu, K. Peters, and T. Vu for their technical assistance, M. Doherty for oligonucleotide synthesis, M. Ando and A. Bell for peptide isolation and sequencing, K. M. Chan for assistance with the computer analyses, and K. Peters, J. Escobedo, S. Coughlin, and P. Orchansky for helpful discussions and critical reading of the manuscript.

18 April 1989; accepted 19 May 1989

Pretranslational Suppression of an Insulin-Responsive Glucose Transporter in Rats with Diabetes Mellitus

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A prominent feature of diabetes mellitus is the inability of insulin to appropriately increase the transport of glucose into target tissues. The contributions of different glucose transport proteins to insulin resistance in rats with streptozotocin-induced diabetes was evaluated. A glucose transporter messenger RNA and its cognate protein that are exclusively expressed in muscle and adipose tissue were specifically depleted in diabetic animals, and these effects were reversed after insulin therapy; a different glucose transporter and its messenger RNA that exhibit a less restricted tissue distribution were not specifically modulated in this way. Depletion of the muscle- and adipose-specific glucose transporter species correlates with and may account for the major portion of cellular insulin resistance in diabetes in these animals.

Desensitization of the glucose transport effector system is a major cause of insulin resistance in clinical disease states and in several cellular models in vitro (*1*). Although insulin has been shown to stimulate glucose transport by inducing a rapid translocation of glucose transporter proteins from a large intracellular pool (associated with low-density microsomes) to the plasma membrane (*2, 3*), the role of glucose transporters in insulin-resistant states has not been elucidated. We have shown that insulin resistance in individuals with type II diabetes mellitus is due in part to depletion of glucose transporters in adipocytes, a classic insulin target tissue (*4*). Similarly, diminished numbers of glucose transporters were found in adipocytes isolated from rats with streptozotocin-induced diabetes mellitus. In this well-characterized animal model, administration of streptozotocin (~50 mg/kg), a pancreatic β cell toxin, induces both stable hyperglycemia (without progression to ketoacidosis) and peripheral insulin resistance (*5, 6*). Nevertheless, the mechanisms underlying transporter depletion in diabetes remain unknown.

A family of glucose transporter genes have been found that have marked differences in their patterns of tissue-specific expression. A cDNA clone has been established from human hepatoma (Hep G2) cells (7), as has its rat homolog from brain (*8*). Two other related genes have also been cloned; a "liver" glucose transporter that is expressed in liver and kidney (*9*) and a "fetal muscle" transporter-like species (*10*). Evidence suggests that these transporter proteins do not mediate the major portion of insulin-stimulated glucose transport activity. First, the amount of the Hep G2 cell (rat brain)–type glucose transporter in adipocyte plasma membranes is increased less than twofold by acute stimulation with insulin, which does not account for the approximately tenfold increment in glucose transport rates. Second, these genes are expressed at low levels or not at all in adult skeletal muscle, which is the most important insulin target tissue for disposal of a glucose load in vivo. The cDNA encoding a related but distinct glucose transporter has been cloned from rat skeletal muscle (*11*). This glucose transporter gene is expressed exclusively in peripheral insulin target tissues (fat, skeletal muscle, and cardiac muscle), and the amount of transporter protein present in adipocyte plasma membranes increases approximately tenfold after acute stimulation with insulin; therefore, this species exhibits characteristics of an insulin-responsive glucose transporter.

To determine mechanisms of transporter depletion and insulin resistance in diabetes, we assessed the impact of streptozotocin-induced diabetes on the expression of different transporter genes in rat adipose and muscle tissue. We specifically studied the insulin-responsive glucose transporter gene cloned from skeletal muscle (*11*) as well as a transporter gene cloned from brain (*8*), as these two genes are expressed in one or more insulin target tissues. Because exogenous insulin therapy has been found to restore the cellular pool of transporters and enhance glucose transport activity in adipocytes (*6*), we also measured the effect of insulin treatment on the expression of these transporter genes.

Despite increased food and water consumption, the streptozotocin-treated rats (14 days after treatment) had diminished body weight and adipocyte size relative to controls and were markedly hyperglycemic (Table 1). In isolated adipocytes, the ability of insulin to maximally stimulate glucose transport rates was reduced by 42% in the diabetic rats, whereas basal glucose trans-

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Table 1. Effect of diabetes mellitus and insulin therapy on body weight, plasma glucose concentration, and adipocyte glucose transport rates. Male Sprague-Dawley rats with initial weights of 163 ± 13 g were either studied as controls or injected in the tail vein with streptozotocin (50 mg/kg) (diabetic). The streptozotocin-treated rats became glycosuric within 48 hours, and both control and diabetic rats were maintained for 2 weeks with free access to food and water. In the second week, a subgroup of diabetic animals was given daily subcutaneous injections of purified pork neutral protamine Hagedorn insulin (5 units) (Tx-diabetic). All three groups of animals were then killed at 2 weeks, and adipocytes were isolated by collagenase digestion of epididymal fat pads. We assumed that adipocytes were spherical and measured mean adipocyte size with an eyepiece micrometer. Basal and maximally insulin-stimulated (50 ng of insulin per milliliter for 30 min at 37°C) rates of 3-*O*-methylglucose transport were determined in intact cells and normalized per cell surface area as described (*17*). Data represent the means ± SEM from four separate experiments, each with 36 animals per group.

Rat group	Weight (g)	Plasma glucose (mg/dl)	Adipocyte size (pl)	3-*O*-Methylglucose transport	
				Basal (pl sec^{-1} mm^{-2})	Maximum (pl sec^{-1} mm^{-2})
Control	274 ± 9	122 ± 5	141 ± 2	1.8 ± 0.9	29 ± 5
Diabetic	201 ± 6	428 ± 33	60 ± 9	3.4 ± 1.6	17 ± 3
Tx-diabetic	261 ± 8	165 ± 17	112 ± 8	1.3 ± 0.5	44 ± 7

port rates were similar to those in control cells, as has previously been described (*5*). All parameters reverted toward normal in a subgroup of the diabetic animals treated with exogenous insulin; however, insulin-stimulated glucose transport rates in adipocytes were enhanced to values 52% greater than those in control animals.

To examine whether the glucose transport system was regulated at the level of transporter gene expression, we isolated total RNA from adipose tissue and skeletal muscle. The RNA was analyzed on RNA blots under conditions for high-stringency hybridization with ^{32}P-labeled cDNAs encoding muscle (*11*) and brain (*8*) glucose transporters (Fig. 1). In adipose tissue, each transporter cDNA hybridized with single ~2.8-kb mRNA species in all treatment groups. The muscle transporter cDNA also recognized a single 2.8-kb mRNA in skeletal muscle (Fig. 1c); however, brain transporter cDNA hybridized at extremely low levels with RNA from this tissue. Each of the cDNAs hybridized only to the mRNA specifically encoded by each gene under our experimental conditions (Fig. 1d). Although the size of the respective mRNAs was not affected by diabetes, it appeared that the number of transcripts corresponding to muscle transporter (but not brain transporter) was decreased in diabetic animals and then increased as a result of insulin treatment.

We performed multiple experiments (similar to that shown in Fig. 1) to better assess effects on the abundance of transporter mRNAs and quantitated specific mRNA on autoradiograms using densitometric analysis. When data were normalized per equal amounts of total RNA, the amount of muscle transporter mRNA was reduced by 59% in adipose tissue and 30% in skeletal muscle from diabetic rats (Fig. 2a). When diabetic animals were treated with insulin, the amount of muscle transporter mRNA did not change in skeletal muscle but rose in adipose tissue to a value 2.1-fold above that in controls. In contrast, diabetes and insulin therapy did not alter the relative amount of brain transporter mRNA in adipose tissue (Fig. 2d).

The normalization of amounts of specific mRNA relative to total RNA would not represent the number of transcripts per cell if perturbations affected the amount of total RNA per cell. To clarify this issue, we measured the amount of RNA in adipose tissue and skeletal muscle and also quantitated DNA as an index of cell number (Table 2). In diabetic rats, the amount of RNA per DNA was decreased by 61% in adipose tissue and 35% in muscle, consistent with the catabolic state, but these values were restored toward normal by insulin treatment. We then normalized the relative abundance of transporter mRNAs per cellular DNA (Fig. 2, b and e); in diabetic rats the amount of muscle transporter mRNA per cell (Fig. 2b) was reduced in both adipose tissue (by 84%) and skeletal muscle (by 52%). Insulin treatment of diabetic animals increased the number of these transcripts by 58% in muscle and 11-fold in adipose tissue to a value 1.8-fold above normal. Thus, the impact of diabetes and insulin therapy on the amount of cellular muscle transporter mRNA was much greater than could be explained by fluctuations in total cellular RNA (Table 2). In adipose tissue from diabetic rats, the amount of brain transporter mRNA per cell (Fig. 2e) was reduced (by 56%) to a lesser extent than the muscle transporter, commensurate with the decline (by 61%) in total RNA per cell, and then rose in insulin-treated diabetic animals, along with total RNA, to a value within ~20% of normal. Thus, changes in brain transporter mRNA were comparable to variations in total cellular RNA and may reflect nonspecific effects on overall RNA synthesis.

To further examine the specificity of transporter mRNA regulation in diabetes, we also measured cellular levels of a nonrelated mRNA species, CHO-B mRNA (*12*), which encodes a structural protein. We then expressed cellular levels of transporter mRNA relative to CHO-B mRNA (Fig. 2, c and f) and again found that the value for muscle transporter mRNA was reduced in

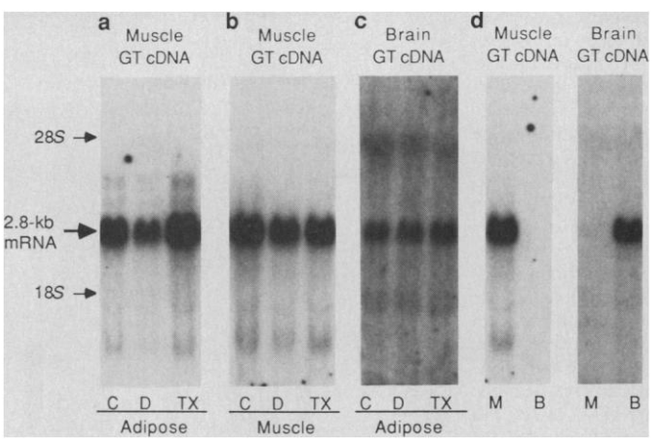

Fig. 1. RNA blot analyses of glucose transporter mRNAs in adipose tissue and skeletal muscle. Groups of control (C), diabetic (D), and insulin-treated diabetic (Tx) rats are described in Table 1. Total RNA was isolated from epididymal fat pads (adipose) and quadriceps femoris (muscle) as described in Table 2. RNA (20 μg) from each group was denatured, size-fractionated on 1% agarose gels, transferred to nylon membranes, and hybridized under high-stringency conditions with ^{32}P-oligolabeled cDNAs (*20*), including a near full-length cDNA (SM1-1) encoding the muscle glucose transporter (*11*) and a near full-length cDNA encoding the brain glucose transporter (*8*). Blots were hybridized in 50% formamide, 2× Denhardt's solution, 1% SDS, 5× standard saline citrate (SSC), and salmon sperm DNA (100 μg/ml) at 42°C. The membranes were washed in 0.2% SDS and 0.1× SSC at 52°C. Autoradiograms of blots from typical experiments are shown. (**a**) Muscle transporter cDNA with RNA from adipose tissue. (**b**) Muscle transporter cDNA with RNA from muscle. (**c**) Brain transporter cDNA with RNA from adipose tissue. (**d**) Muscle and brain transporter cDNAs with RNA from muscle (M) and brain (B). The size of the mRNA species specifically hybridizing with each cDNA was 2.8 kb, and the small arrows show the migration of the 28*S* and 18*S* ribosomal subunits.

diabetic animals and increased after insulin therapy. In contrast, the value for brain transporter mRNA per CHO-B mRNA did not change since cellular levels of both mRNA species fluctuated with changes in total RNA as a function of diabetes and insulin treatment. Thus, muscle transporter mRNA was specifically regulated in insulin target tissue, whereas brain transporter mRNA did not fluctuate independently from CHO-B mRNA or total RNA.

Regulatory effects on glucose transporter mRNA expression may cause insulin resistance by leading to decreased synthesis of transporter proteins in insulin target tissues. Therefore, we measured the amounts of different transporters on protein blots with antiserum specific for the COOH-terminal portion of the muscle transporter (*11*) and with a monoclonal antibody to purified human erthrocyte glucose transporters (*13*), which are encoded by the cDNA cloned from Hep G2 cells (*7*). We studied plasma membrane (PM) and low-density microsomal (LDM) fractions prepared from both basal and maximally insulin-stimulated adipocytes (Fig. 3). In all animal subgroups, acute insulin stimulation led to an increase in the number of transporters in the PM and depletion of transporters in the LDM, indi-

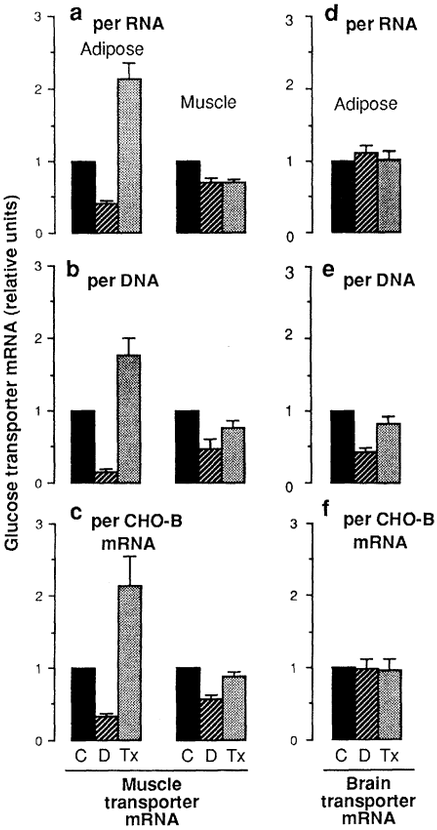

Fig. 2. Effects of diabetes mellitus and insulin therapy on amounts of glucose transporter mRNAs in adipose tissue and skeletal muscle. Groups of control (C), diabetic (D), and insulin-treated diabetic (Tx) rats, preparation of total RNA from tissues, and RNA blot analyses are described in Table 1 and Fig. 1. Specific mRNA was quantitated from autoradiograms by laser-scanning densitometry relative to the amount in controls, which was assigned a value of 1. The relative amounts of mRNA hybridizing with muscle transporter cDNA (*11*) and with brain transporter cDNA (*8*) were normalized as follows: per equal amounts of total RNA from each group in (**a**) and (**d**); per tissue DNA content as an index of cell number in (**b**) and (**e**); and each transporter mRNA per DNA was normalized for the amount of CHO-B mRNA per DNA in (**c**) and (**f**). Methods for measuring tissue levels of RNA and DNA and for calculating the mean values in each group are given in Table 2. Full-length CHO-B cDNA (*12*) hybridized with a single 1.1-kb mRNA in adipose and muscle tissue from all treatment groups. Hybridization of brain transporter cDNA with RNA from muscle was too low to quantitate accurately by RNA blot analysis. The data represent the means ± SEM from four separate groups of animals, each with 36 animals per group.

Table 2. Effects of diabetes mellitus and insulin therapy on tissue content of RNA and DNA. Groups of control, diabetic, and insulin-treated diabetic rats were established as described for Table 1. After rats were killed, epididymal fat pads and quadriceps femoris skeletal muscle tissue were removed and pooled within each group, and total RNA was isolated (*18*). Quantity and purity of RNA was assessed by absorbance at 260 and 280 nm; the mean value for the absorbance 260/280 ratio was 1.7 in all treatment groups in both adipose tissues and skeletal muscle. For measurements of DNA, fat and muscle tissue were immediately frozen in liquid nitrogen, and DNA was quantitated later in crude homogenates by a spectrofluorometric assay (*19*). Data represent the means ± SEM from four separate groups of animals, each with 36 animals per group.

Rat group	Adipose tissue			Skeletal muscle		
	RNA (μg/g)	DNA (mg/g)	RNA/DNA (μg/mg)	RNA (μg/g)	DNA (mg/g)	RNA/DNA (μg/mg)
Control	118 ± 27	0.63 ± 0.03	282 ± 43	362 ± 43	1.86 ± 0.05	195 ± 18
Diabetic	220 ± 35	2.00 ± 16	110 ± 17	276 ± 48	2.20 ± 0.09	126 ± 22
Tx-diabetic	263 ± 42	1.16 ± 0.08	227 ± 36	375 ± 10	1.86 ± 0.08	202 ± 5

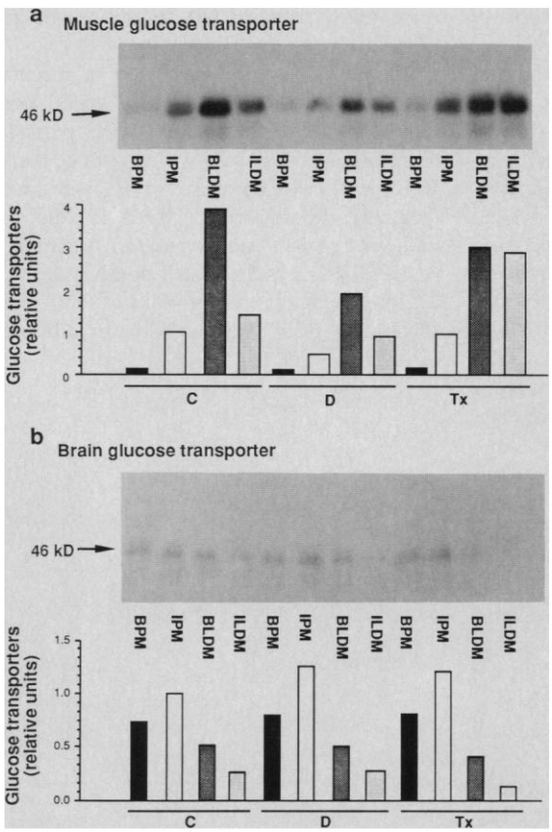

Fig. 3. Effects of diabetes and insulin therapy on glucose transporters in adipocyte membrane subfractions. Groups of control (C), diabetic (D), and insulin-treated diabetic (Tx) rats are described in Table 1. When rats were killed, epididymal fat pads were pooled within each subgroup and isolated adipocytes were prepared by collagenase digestion (1 mg/ml for 1 hour at 37°C) of the tissue. The cells were incubated in the absence and presence of a maximal insulin concentration (100 ng/ml for 30 min at 37°C) and then homogenized. The subcellular distribution of glucose transporters was investigated by the use of a differential ultracentrifugation scheme to prepare plasma membrane (PM) and low-density microsomal (LDM) membrane subfractions from both basal (B) and insulin-stimulated (I) cells. Preparation of adipocytes and the isolation and characterization of subcellular membrane fractions were as described (*4, 17*). Equal amounts of membrane protein were solubilized in a Laemmli sample buffer and analyzed by SDS–polyacrylamide gel electrophoresis on 1.5-mm slab gels containing 10% polyacrylamide. The proteins were then electrophoretically transferred to nitrocellulose (*21*). (**A**) Immunological detection of the glucose transporter with affinity-purified antiserum specific for the COOH-terminal portion of the rat skeletal muscle transporter (*11*). Membrane protein (30 μg) was loaded on each lane of the gel, and cross-reacting protein was detected with ^{125}I-labeled protein A (*22*) and autoradiography. (**B**) Immunological detection of glucose transporters with mouse monoclonal antibody F-18 (*13*) that was raised against purified human erythrocyte glucose transporters. Membrane protein (75 μg) was loaded on each lane of the gel, and cross-reacting protein was measured with an alkaline phosphatase–linked antibody to mouse immunoglobulin G (ProtoBlot system, Promega), and scanning densitometry. In both panels, mean numbers of transporters are given as a percentage of the value (densitometry units) for IPMs from control animals [(A) $n = 4$, (B) $n = 2$]. Individual values were consistently within 9% of the mean value.

cating that a translocation of transporters from cell interior to cell surface was induced; this effect was observed for both species of transporters. However, muscle transporters in PM were increased eightfold in response to insulin, compared with an ~1.5-fold increment for brain transporters. There was a differential effect of diabetes on the cellular content of each transporter protein. Muscle glucose transporters (per milligram of protein) were decreased by ~50% in both membrane subfractions from diabetic rats and were restored toward normal after insulin therapy. In contrast, brain transporters were not affected by diabetes or insulin treatment in any of the subfractions.

In a manner analogous to effects on cellular RNA, the amount of protein per cell in all membrane subfractions was reduced by 30 to 42% in diabetic rats (*5, 14*). Therefore, the actual reduction in the number of muscle transporters per cell was greater than that estimated by the protein blot analysis (Fig. 3a), in which equal amounts of protein were loaded in each lane. Also, insulin treatment of diabetic animals led to ~60% increase in cellular protein (*6, 14*), and therefore the protein blot analysis (Fig. 3a) represents underestimates of therapy-induced increments in muscle transporters per cell. Thus, the effects on muscle transporter mRNA correlated well with changes in the encoded protein, and pretranslational mechanisms may predominate in regulating the number of these glucose transporters in diabetes.

Karnieli *et al.* (*5*) found that total cellular glucose transporters are decreased by ~50% in adipocytes from streptozotocin-treated diabetic rats when measured by the cytochalasin B–binding assay (*3*), which identifies all species of glucose transporters. Our data now allow an estimate of the relative contribution of different species to overall transporter depletion. The selective diminution in the muscle transporter correlates well with the diabetes-induced depletion in cytochalasin B binding and may account for the major portion of transporter loss. Furthermore, changes in the amount of the muscle transporter may explain impaired insulin responsiveness in diabetic animals, suggesting that this transporter predominates in facilitating insulin-responsive glucose transport. These hypotheses are also supported by observations that cellular cytochalasin B–binding sites, insulin-stimulated glucose transport rates, and muscle glucose transporters are all increased after insulin therapy. It seems likely that brain glucose transporters mediate most of the basal glucose uptake, as both basal rates and brain transporters are not specifically regulated in these experimental animals.

Our data have implications regarding the pathogenesis of insulin resistance in individuals with diabetes mellitus. In type II (non-insulin-dependent) diabetes, the impaired ability of insulin to promote glucose uptake in target tissues is largely due to cellular depletion of glucose transporters (*1, 4*), and institution of therapy leads to partial reversal of insulin resistance, associated with enhanced activity of the glucose transport system (*1, 15*). Similarly, poorly controlled patients with type I (insulin-dependent) diabetes exhibit decreased insulin responsiveness for stimulation of glucose uptake that can be normalized after intensive insulin therapy (*16*). On the basis of our data, insulin resistance in diabetes and its amelioration with therapy could be due to changes in the amounts of muscle glucose transporter mRNA and protein contained in cells. Thus, decreased numbers of these transporters in target tissue may constitute a predominant biochemical lesion that maintains the diabetic state.

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22. We thank J. Vinten for the antibody to the erythrocyte glucose transporter and R. M. Evans for the cDNA encoding CHO-B. The work was supported by NIH grants DK 38765 and DK 39519 and in part by a gift from the Lucille P. Markey Charitable Trust. W.T.G. is the recipient of a Pfizer Scholars Award and a Research and Development Award from the American Diabetes Association. We would also like to thank J. P. Rosselle for manuscript preparation.

24 February 1989; accepted 9 May 1989

Human Diabetes Associated with a Deletion of the Tyrosine Kinase Domain of the Insulin Receptor

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The insulin receptor has an intrinsic tyrosine kinase activity that is essential for signal transduction. A mutant insulin receptor gene lacking almost the entire kinase domain has been identified in an individual with type A insulin resistance and acanthosis nigricans. Insulin binding to the erythrocytes or cultured fibroblasts from this individual was normal. However receptor autophosphorylation and tyrosine kinase activity toward an exogenous substrate were reduced in partially purified insulin receptors from the proband's lymphocytes that had been transformed by Epstein-Barr virus. The insulin resistance associated with this mutated gene was inherited by the proband from her mother as an apparently autosomal dominant trait. Thus a deletion in one allele of the insulin receptor gene may be at least partly responsible for some instances of insulin-resistant diabetes.

TYROSINE-SPECIFIC PROTEIN KINASES participate in many cellular signal transduction events. The products of several oncogenes and certain growth factor receptors are tyrosine-specific protein kinases (*1*). The insulin receptor β subunit has an intrinsic tyrosine kinase activity that is activated by the binding of insulin to the