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Purification and Complementary DNA Cloning of a Receptor for Basic Fibroblast Growth Factor

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Basic fibroblast growth factor (bFGF) participates in many processes including early developmental events, angiogenesis, wound healing, and maintenance of neuronal cell viability. A 130-kilodalton protein was isolated on the basis of its ability to specifically bind to bFGF. A complementary DNA clone was isolated with an oligonucleotide probe corresponding to determined amino acid sequences of tryptic peptide fragments of the purified protein. The putative bFGF receptor encoded by this complementary DNA is a transmembrane protein that contains three extracellular immunoglobulin-like domains, an unusual acidic region, and an intracellular tyrosine kinase domain. These domains are arranged in a pattern that is different from that of any growth factor receptor described.

THE FIBROBLAST GROWTH FACTOR (FGF) family consists of polypeptide growth factors characterized by amino acid sequence homology, heparin-binding avidity, the ability to promote angiogenesis, and mitogenic activity toward cells of epithelial, mesenchymal, and neural origin. Members of the FGF family appear to have roles in development, tissue repair, maintenance of neurons, and the pathogenesis of disease (*1–6*). Aberrant expression of FGFs may cause cell transformation by an autocrine mechanism (*7, 8*). Moreover, FGFs may enhance tumor growth and invasiveness by stimulating blood vessel growth into the tumor (*4*) or by inducing production of proteases such as plasminogen activator (*9*).

The FGF family includes acidic and basic FGFs (*10*), the *int-2* gene product (*11*), the *hst* gene product (Kaposi sarcoma–FGF) (*6, 12*) FGF-5 (*13*), and the keratinocyte growth factor (*14*). The actions of acidic and basic FGF are mediated through binding to high-affinity cell surface receptors of 145 and 125 kD (*15*). It is not known, however, whether each FGF interacts with a different receptor or whether the different forms of FGF share the same receptor. We now describe an attempt to characterize the primary structure of the receptor for basic FGF (bFGF).

The bFGF receptor was purified by a lectin-affinity chromatography step followed by a specific ligand-affinity chromatography procedure with bFGF that had been biotinylated on cysteine residues. The modified bFGF was indistinguishable from unmodified bFGF in its ability to inhibit the binding of ^{125}I-labeled bFGF to high-affinity bFGF receptors in Swiss 3T3 cells (Fig. 1A) and its ability to stimulate the phosphorylation of a 90-kD protein (*16*), known to be a substrate of bFGF-induced tyrosine kinase activity (*17*). The biotinylation reaction modified 90 to 95% of the bFGF molecules as measured by binding to avidin-conjugated agarose (Fig. 1A). Less than 5% of control bFGF, which had been subjected to a mock biotinylation reaction, bound to the avidin-agarose (Fig. 1A). Iodine-125–labeled biotin-bFGF bound to bFGF receptors in Swiss 3T3 cells with high affinity (dissociation constant = 1 n*M*) and could

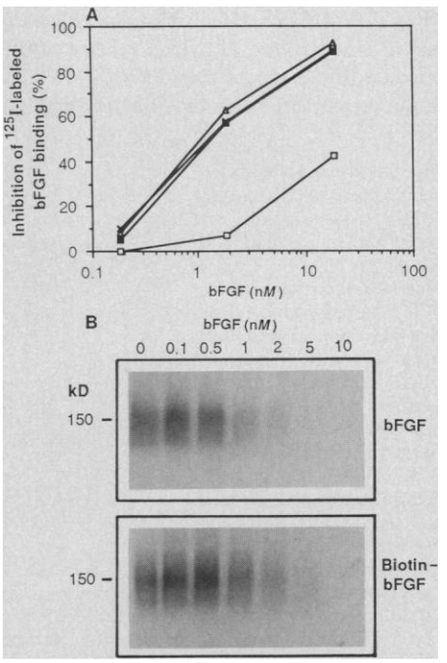

Fig. 1. (**A**) Competitive binding of ^{125}I-labeled bFGF to Swiss 3T3 cells. Iodine-125–labeled bFGF (2 Ci/μmol) was added to confluent cells (6 fmol of ^{125}I-labeled bFGF per 10^5 cells) in the presence of the indicated concentrations of: unmodified bFGF (x); biotin-bFGF (■); the unbound fraction after biotin-bFGF was incubated with avidin-agarose (□); the unbound fraction after bFGF was incubated with avidin-agarose (△). Binding was performed for 30 min at 37°C in culture media (DME H21) containing 0.2% gelatin and heparin (15 U/ml). The cells were washed three times with a buffer containing 20 m*M* Hepes (*p*H 7.4), 0.2% gelatin, and 150 m*M* NaCl. The radioactivity present was determined in a Beckman gamma counter. Maximal binding (0% inhibition) represents 5700 cpm of specific binding (nonspecific binding was 600 cpm). All determinations were made in triplicate. Recombinant human bFGF (*37*) was iodinated using iodogen (Pierce). bFGF was biotinylated with iodoacetyl-LC-biotin (Pierce) at a 4:1 molar excess of cysteine residues in 10 m*M* tris-HCl (*p*H 8.0) for 5 hours at 4°C (*38*). Unreacted biotin was removed by gel filtration with PD 10 columns (Pharmacia). (**B**) Whole-cell cross-linking. ^{125}I-labeled biotin-bFGF or ^{125}I-labeled bFGF (0.1 pmol) was added to Swiss 3T3 cells (5×10^5 cells) in the presence or absence of unlabeled bFGF as indicated. The cells were washed and cross-linked with 0.15 m*M* disuccinimidyl suberate (DSS) (Pierce). The cells were then solubilized and subjected to SDS–polyacrylamide gel electrophoresis (PAGE), and ^{125}I-labeled proteins were detected by autoradiography.

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be cross-linked to a 130-kD protein, which comigrated with the bFGF receptor cross-linked to ^{125}I-labeled bFGF (Fig. 1B).

Chicken embryos (day 6, stage 29 to 30) were used as our source of bFGF receptor because they contain relatively large amounts of the protein as determined by high-affinity binding of human and bovine bFGF (*16, 18*). Specific binding and cross-linking of ^{125}I-labeled bFGF to a crude membrane fraction of chicken embryos revealed only a single protein band of 150 kD (Fig. 2A). After the molecular mass of bFGF is subtracted, the deduced size of the chicken bFGF receptor is 130 to 135 kD. Embryo extracts were first fractionated on wheat germ agglutinin (WGA)–Sepharose 4B (*19*), and the partially purified bFGF receptors (Fig. 2A) were then bound to biotin-bFGF and adsorbed to avidin-agarose. The nonspecific binding of proteins to avidin-agarose was assessed by a parallel incubation of the receptor-containing fraction with avidin-agarose in the absence of biotin-bFGF. The avidin-agarose columns were eluted with suramin, which dissociates bFGF from its receptor (*17*). Although a number of proteins bound to avidin-agarose in a nonspecific manner, only a single protein bound to avidin-agarose in an FGF-dependent manner (Fig. 2B). This protein migrated with the expected size (130 kD) of the bFGF receptor (Fig. 2B).

Two independent purifications were performed, yielding 20 and 50 μg of the 130-kD protein. The protein was digested with trypsin, and peptide fragments were separated by reversed-phase high-performance liquid chromatography (HPLC) and analyzed by gas-phase sequencing (*20*). From the two independent preparations, we obtained the amino acid sequences of 14 peptides. Three of the peptides were common to both preparations, indicating the identity of the two independent preparations. The sequence of the NH_2-terminus of the 130-kD protein could not be determined. Four of the tryptic peptides (LILGKPLGEGCFG-QVVLA, IADFGLAR, MAPEALFDR, and IYTHQSDVWSFGV) (*21*) were homologous to consensus sequences for tyrosine kinase domains (Fig. 3). This homology was consistent with the finding that tyrosine kinase activity is associated with the bFGF receptor (*17, 22*). Thus, the protein we purified bound to bFGF, had the expected molecular mass of the receptor, and contained tyrosine kinase amino acid sequences.

The amino acid sequences of 11 of the 14 peptides were identified as being present in a partial human cDNA clone, termed *flg* (*fms*-like gene) (*23*). The *flg* clone was isolated on the basis of its homology to the *fms* proto-oncogene sequence and was not previously recognized as encoding a transmembrane receptor protein (*23*). A full-length cDNA clone of the chicken bFGF receptor was isolated with an oligonucleotide probe based on the amino acid sequences of two tryptic peptides following the codon usage of the published *flg* cDNA sequence. The oligonucleotide probe was used to screen a chicken embryo (day 6) cDNA library under low-stringency conditions, and 11 positive clones were isolated. The deduced amino acid sequence of the largest clone (3.2 kb) contained the sequence of all 14 of the receptor peptides (Fig. 3).

A single hybridizing band of approximately 3.5 kb was identified by RNA-blot analysis of chicken embryo polyadenylated RNA with the cDNA clone of the putative receptor (Fig. 4A). Also, primer-extension experiments with an oligonucleotide complementary to the 5′ end of the clone predicted that the mRNA of the receptor was 48 nucleotides longer than the isolated clone (Fig. 4B).

Analysis of the amino acid sequence of the longest open reading frame (2.4 kb) revealed an in-frame stop codon (residue −12) followed by an initiator methionine (*24*) and the entire putative receptor coding sequence. The cDNA encoded a protein with a deduced molecular mass of 91.7 kD

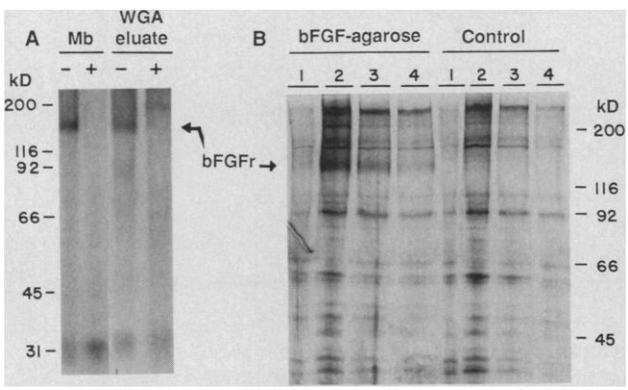

Fig. 2. (**A**) Cross-linking of the chicken bFGF receptor (bFGFr). Cross-linking was performed by incubating 10 μl of a chicken embryo membrane fraction (Mb) or 100 μl of the eluate from the WGA-Sepharose 4B column (*19*) with ^{125}I-labeled bFGF (0.1 pmol) in the presence (+) or absence (−) of a 200-fold excess of unlabeled bFGF for 30 min at 37°C. DSS was added to a concentration of 0.15 m*M*, and the reaction mixture was incubated for 10 min on ice. Samples were subjected to SDS-PAGE followed by autoradiography. (**B**) Ligand affinity chromatography. Two large-scale affinity purifications were performed (each using the material from 20,000 embryos). The eluate from the WGA-Sepharose 4B column was incubated with biotin-bFGF (10:1 molar excess of ligand to receptor) and heparin at a concentration of 15 U/ml (to reduce low-affinity binding) (*39*) for 30 min at 4°C. The mixture was then cycled twice through a 10-ml avidin-agarose column (bFGF-agarose). Alternatively, the eluate from the WGA-Sepharose 4B column was cycled through avidin-agarose in the absence of biotin-bFGF (control). The columns were washed with 200 ml of column buffer (*19*) containing 0.2*M* NaCl followed by 300 ml of column buffer without NaCl and then eluted with 10 m*M* suramin in column buffer. Four sequential 10-ml fractions were collected and samples of each fraction were subjected to SDS-PAGE and stained with silver nitrate. This procedure yielded 2 to 5 ng of pure FGF receptor per chicken embryo with an overall recovery of 5%.

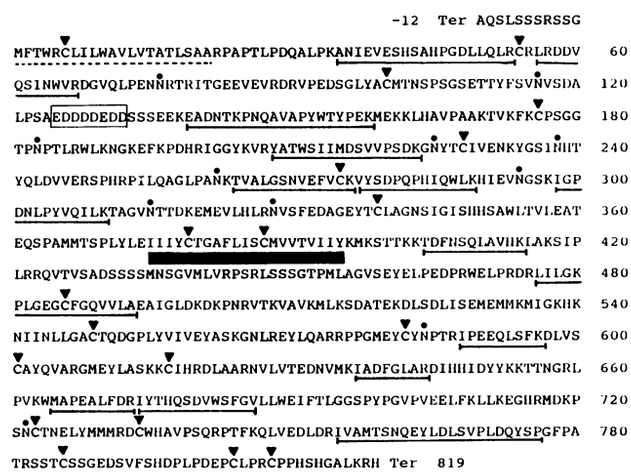

Fig. 3. Amino acid sequence of chicken bFGF receptor. A chicken embryo (day 6) cDNA library containing 2×10^6 recombinants was generated from size-fractionated polyadenylated RNA (>2.5 kb) and screened with ^{32}P-labeled oligomers that encoded the peptides TVALGSNVEFV-CK and VYSDPQPHIQW-LK (*21*). Filters were hybridized under low-stringency conditions [20% formamide, 5× standard saline citrate (SSC), and 5× Denhardt's solution at 42°C] and washed with 0.2× SSC at 42°C. The amino acid sequence of the cDNA clone with the longest open reading frame is shown. In the absence of the NH_2-terminal amino acid sequence, we have assigned the initiator methionine as the first residue. The transmembrane region was identified by Kyte and Doolittle hydropathy analysis (*40*). Peptides sequenced from the purified protein (underlined); transmembrane sequence (heavy underline); acidic region (open box); and putative hydrophobic leader sequence (dashed underline), cysteine residues (▼); potential N-linked glycosylation sites (superior dot). The nucleotide sequence has been submitted to GenBank.

that had features found in several growth factor receptors. It contained a single membrane-spanning region, an NH_2-terminal hydrophobic leader sequence, three extracellular immunoglobulin-like domains, and an intracellular tyrosine kinase domain (Fig. 5). Eleven potential N-linked glycosylation sites were also found. N- and O-linked glycosylation of the chicken bFGF receptor may account for the disparity between the observed size of the bFGF receptor and the size predicted from the cDNA sequence.

Three immunoglobulin-like domains in the putative extracellular region were identified on the basis of three criteria (*25*): (i) the presence of two characteristic cysteine residues in each domain; (ii) the presence of a consensus tryptophan residue 11 or 12 amino acids on the COOH-terminal side of the first cysteine residue in each immunoglobulin-like domain; and (iii) the presence of the consensus sequence DXGXYXC (*21*) on the NH_2-terminal side of the second cysteine residue. The interleukin-1 (IL-1) receptor also has three immunoglobulin-like domains, and the amino acid sequence of bFGF is 25 to 30% identical to that of IL-1 (*26, 27*). Five immunoglobulin-like domains are present in the receptors for platelet-derived growth factor (PDGF) (*20*) and colony-stimulating factor–1 (CSF-1) (*28*).

Between the first and second immunoglobulin-like domains, the bFGF receptor has a feature not found in other members of the immunoglobulin superfamily. There is a series of eight consecutive acidic residues (EDDDDEDD) (*21*) followed by three serine residues and two additional acidic residues. Although uninterrupted stretches of 7 to 35 acidic residues have been described for several intracellular proteins, in particular nuclear proteins (*29*), such acidic regions are unusual in the extracellular region of transmembrane receptor proteins.

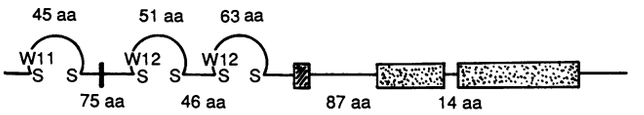

Fig. 5. Schematic of chicken bFGF receptor. Shown in the diagram are the acidic region (black box); transmembrane region (hatched box); tyrosine kinase region (dotted boxes); S, position of cysteine residues; W, position of tryptophan residue with respect to the first cysteine residue in the immunoglobulin-like domain. Also indicated are the number of amino acids (aa) in each region.

Another unusual feature is the length of the juxtamembrane region, the region between the membrane-spanning segment and the kinase domain. This region is normally conserved among receptor tyrosine kinases. For example, the juxtamembrane region is consistently 49 to 51 residues in length in the receptors for PDGF (*20*), CSF-1 (*28*), epidermal growth factor (EGF) (*30*), human epidermal growth factor–2 (HER2) (*31*), and insulin (*32*). The bFGF receptor has an unusually long juxtamembrane region of 87 residues.

The cytoplasmic region of the predicted amino acid sequence is 424 residues long and contains a tyrosine kinase sequence (residues 483 to 759). Overall, the kinase region of the bFGF receptor shares the most sequence identity (51 to 53%) with the PDGF and CSF-1 receptors (*33*). The bFGF receptor contains the GXGXXG (*21*) motif and the conserved lysine residue (residue 512) that form part of the adenosine triphosphate (ATP) binding site of tyrosine kinases. The bFGF receptor also contains the two characteristic tyrosine kinase motifs HRDLAARNVL and DFGLAR (*21*), and a tyrosine (residue 651) at the position analogous to the major phosphorylation site of $pp60^{v\text{-}src}$ (Tyr^{416}).

The kinase sequence of the bFGF receptor, defined by homology to other tyrosine kinases, is split by an insertion of 14 amino acids. The length of the insertion in the kinase region is much shorter than that found in the receptors for PDGF (*20*) and CSF-1 (*28*) (104 and 70 amino acids, respectively) and is similar to the length of the inserted sequence in the receptors for insulin and insulin-like growth factor–1 (*32, 34*).

Using oligonucleotide probes, we have isolated a bFGF receptor cDNA clone from a human endothelial cell cDNA library (*35*). Between the chicken and human bFGF receptors, there is 98 to 100% amino acid sequence identity in the kinase region, 86% in the transmembrane region, 92% in the juxtamembrane region, 79% in the kinase insert region, and 80% in the COOH-terminal region.

The chicken bFGF receptor has amino acid sequence similarity to two previously identified partial cDNA clones. They are the mouse tyrosine kinase gene, *bek* (84% sequence identity) (*36*), and, as mentioned earlier, human *flg*, localized on chromosome 8p12 (*23*). As only the kinase domain of *bek* has been identified, information on the NH_2-terminal region is required to determine whether this gene encodes the mouse bFGF receptor or a closely related receptor. The cDNA clone of human *flg* was obtained by low-stringency screening of an endothelial cell cDNA library with the use of human c-*fms* cDNA as a probe (*23*). Although only a partial cDNA sequence has been reported for *flg* (*23*), it seems likely that *flg* encodes the human bFGF receptor.

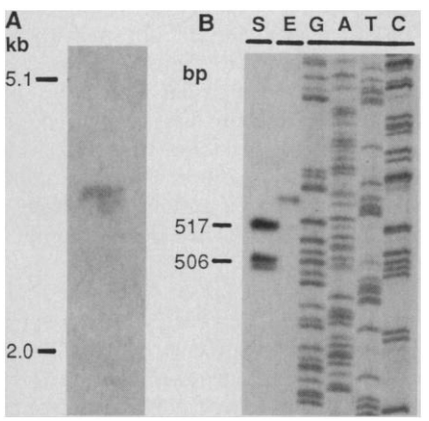

Fig. 4. (**A**) RNA blot of chicken RNA. Chicken embryo polyadenylated RNA (5 μg) was probed with full-length chicken bFGF receptor cDNA under high-stringency conditions (50% formamide, 5× Denhardt's solution and 5× SSC at 42°C). Filters were then washed with 0.2× SSC at 65°C. (**B**) Primer extension. Chicken embryo polyadenylated RNA (5 μg) was denatured with 10 m*M* methylmercury, annealed with ^{32}P-labeled primer (5′-CTGCACGTCATCGCGCA-3′), and extended with murine Moloney leukemia virus reverse transcriptase. Lane S, ^{32}P-labeled DNA molecular size standards (1-kb ladder); lane E, extended fragment (523 nucleotides); lanes GATC, 5% acrylamide sequencing gel.

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19. Fresh, day-6 chicken embryos (1500 per batch) were homogenized (1:1 v/v) in a final concentration of 0.25*M* sucrose, 50 m*M* Hepes (*p*H 7.5), 2 m*M* EDTA, 50 m*M* NaF, 150 μ*M* sodium orthovanadate, 30 m*M* sodium pyrophosphate, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), aprotinin [20 to 30 kallikrein inactivator units (KIU) per milliliter], leupeptin (10 μg/ml), and pepstatin (1 μg/ml) with

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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21. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln, R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; X, any amino acid.
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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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