High-level expression of human insulin receptor cDNA in mouse NIH 3T3 cells

(bovine papilloma virus/glycosylation/biosynthesis/glycogen synthesis)

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ABSTRACT In order to develop a simple, efficient system for the high-level expression of human insulin receptors in eukaryotic cells, a full-length human kidney insulin receptor cDNA was inserted into a bovine papilloma virus vector under the control of the mouse metallothionein promoter. After transfection of mouse NIH 3T3 cells with this construct, seven cell lines expressing insulin receptors were isolated; two cell lines had more than 10^6 receptors per cell. The cell line with the highest insulin binding (NIH 3T3 HIR3.5) had 6×10^6 receptors with a K_d of 10^{-9} M. This level was not dependent on exposure to metals but could be increased further to 2×10^7 receptors per cell by addition of sodium butyrate to the culture medium. The α and β subunits had apparent molecular weights of 147,000 and 105,000, respectively (compared to 135,000 and 95,000 in IM-9 human lymphocytes), values identical to those of the α and β subunits of the insulin receptors of nontransformed NIH 3T3 cells. This size difference was due to altered carbohydrate composition, as N-glycanase digestion reduced the apparent receptor subunit size of the transfected cells and IM-9 lymphocytes to identical values. The alteration in N-linked oligosaccharide composition could not be ascribed to differences in the kinetics of posttranslational processing of the insulin receptors, which was comparable to that of other cells studied. The basal rate of glycogen synthesis in the cells overexpressing insulin receptors was increased 4- to 5-fold compared with controls. Low levels of added insulin (0.1 nM) caused a 50% increase in the rate of glycogen synthesis.

The insulin receptor is a glycoprotein composed of two α subunits and two β subunits linked by disulfide bonds. The α subunit is located exclusively extracellularly and contains the major insulin-binding domains (for review see ref. 1). The isolation and sequencing of the human placental insulin receptor cDNA (2, 3) have furnished primary structural information about the insulin proreceptor protein. The 755 amino acid α subunit represents the amino-terminal portion of the predicted 1382 amino acid precursor and contains a striking cysteine-rich domain. This, by analogy with the low density lipoprotein receptor (4), may be the ligand-binding domain. The cloning and sequencing of the human insulin-like growth factor I receptor (5)—a related protein with identical subunit structure and a similar assignment of functional domains (6, 7)—have provided further insights into the potential location of the ligand-binding domains of both receptors. Thus, differences in ligand specificities are likely to be determined by sequence differences in the α subunits; low homology is found in the extracellular cysteine-rich domain (48%) and in the carboxyl-terminal one-third (47%). More detailed characterization of the insulin-binding do-

main of the insulin receptor by protein-chemical techniques and site-directed mutagenesis will require the development of a system for overexpression of human insulin receptor cDNAs in eukaryotic cells. This has recently been achieved using a simian virus 40-based expression system in CHO cells (8). However, in this study it was necessary to use fluorescence-activated cell sorting and cell cloning to isolate cell lines expressing 10^5 – 10^6 receptors per cell.

To simplify the selection of overexpressing clones for studies of mutated receptor sequences, we have investigated the use of a vector based on bovine papilloma virus (BPV) for overexpression of human insulin receptor cDNAs. Cells transformed with vectors containing the entire BPV genome or a 5.5-kilobase (kb) subgenomic transforming fragment invariably carry many copies of the gene of interest and express foreign proteins very efficiently (for review see ref. 9). This paper describes the derivation and characterization of a cell line that expresses insulin receptors at very high levels from a human kidney insulin receptor cDNA BPV vector construct.

MATERIALS AND METHODS

General. Preparation of plasmid DNA, agarose gel electrophoresis, transfer of DNA to nitrocellulose, hybridization, radiolabeling of DNA by nick-translation or end-labeling, repair of DNA, ligation, addition of synthetic linkers, bacterial transformation, and DNA sequencing were performed by standard methods (10, 11). Enzymes were from Boehringer Mannheim, New England Biolabs, or Pharmacia P-L, except where specified. All oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) 380B DNA synthesizer. Radiochemicals, including insulin radioiodinated at tyrosine-14 of the A chain ($[^{125}\text{I-Tyr-A14}]$ insulin) or tyrosine-26 of the B chain ($[^{125}\text{I-Tyr-B26}]$ insulin), were purchased from Amersham. Protease inhibitors were from Boehringer Mannheim. Media and serum for tissue culture were from Irvine Scientific.

Isolation of Human Kidney Insulin Receptor cDNAs. Two insulin receptor cDNA clones, 18-2 and 13-1, were isolated from a human kidney cDNA library constructed in bacteriophage λ gt10 (12) by screening with synthetic oligonucleotide probes. DNA sequencing showed 18-2 to commence at the first guanine of the initial glycine codon of the signal peptide and to extend to the internal *EcoRI* site (2, 3) and 13-1 to extend from the latter *EcoRI* site into the 3' untranslated region. The predicted amino acid sequence was that determined by Ebina *et al.* (3).

Abbreviations: BPV, bovine papilloma virus; $[^{125}\text{I-Tyr-A14}]$ insulin, insulin radioiodinated at tyrosine-14 of the A polypeptide chain; $[^{125}\text{I-Tyr-B26}]$ insulin, insulin radioiodinated at tyrosine-26 of the B polypeptide chain.

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Oligonucleotide-Directed Mutagenesis. To add the consensus initiation sequence ACCATG (13) to the 5' end of 18-2, cDNA 18-2 was cloned into the *EcoRI* site of bacteriophage vector M13 mp11 and the noncoding strand was used as a template for oligonucleotide-directed mutagenesis (14), with a 40-mer oligodeoxynucleotide (CGCCGGCCCCCGGTGCCATGGTACGAGCTCGCCCGGGGA) as the mutagenic primer and the universal M13 sequencing primer as the nonphosphorylated primer. After confirmation of the mutation by DNA sequencing, the double-stranded replicative form (RF) of the mutant M13 clone was prepared. As the *EcoRI* linker sequences at the 5' end of 18-2 were eliminated during mutagenesis, the modified 18-2 cDNA insert was excised from the M13 RF by digestion with *Sal I* (at the M13 mp11 polylinker *Sal I* site) and *EcoRI*.

Construction of Full-Length Human Insulin Receptor cDNA and BPV Recombinant. The mutated 18-2 cDNA was subcloned into *Sal I*/*EcoRI*-digested pAT153 (32). After redigestion with *EcoRI*, the 3' human insulin receptor cDNA 13-1 was ligated into it. The resultant plasmid, designated pHIR, was then digested with *Asu II* (Promega Biotec, Madison, WI), which cuts the human insulin receptor cDNA at a single site in the 3' untranslated region [nucleotide 4705 according to Ebina *et al.* (3)]. After addition of synthetic *Sal I* oligonucleotide linkers (Pharmacia P-L) and *Sal I* digestion, the 4.6-kb fragment, containing the entire insulin receptor cDNA coding region, was ligated into the *Xho I* site of pBPV-MTHXho (kindly provided by D. Hamer, National Institutes of Health). This vector is similar to others described by Pavlakis and Hamer (15). It contains the 5.5-kb subgenomic transforming fragment of BPV, the origin of replication and the ampicillin-resistance gene of pML (16), and the mouse metallothionein gene (17) modified by replacement of the *Bgl II* site upstream of the metallothionein gene initiator ATG with an *Xho I* site. The resulting construct, pBPVHIRA⁻, is shown in Fig. 1.

Cell Transfection and Selection of Transformants. NIH 3T3 cells ($2-4 \times 10^5$) were cotransfected with 5 μ g of a calcium phosphate precipitate of pBPVHIRA⁻ and the plasmid pSV2neo (18) in a 10:1 ratio and selected by culture in the presence of G418 (GIBCO) at 600 μ g/ml as described by Gorman (19). After 3 weeks, G418-resistant colonies were isolated and grown in 12-well tissue culture plates.

Insulin Binding Assays. NIH 3T3 cells were plated in 24-well culture dishes at $2-4 \times 10^3$ cells per well. Two days later, cells were washed twice with binding buffer [Hanks' balanced salts solution supplemented with 50 mM Hepes (pH 7.6) and 10 mg of bovine serum albumin per ml] and then incubated as described in the text. Wells were then aspirated, and the cells were washed three times with 1 ml of ice-cold

phosphate-buffered saline prior to solubilization in 500 μ l of 0.1 M NaOH for 60 min at 37°C for measurement of radioactivity.

Solubilization and Immunoprecipitation. Cells were solubilized for 30 min at 4°C in 200 mM NaCl/50 mM Tris-HCl, pH 8.0/2% (vol/vol) Nonidet P-40/0.1% NaDodSO₄ containing phenylmethanesulfonyl fluoride (0.2 mM), pepstatin (0.5 μ g/ml), and leupeptin (0.5 μ g/ml) (1 ml per 100-mm dish). After removal of insoluble material by centrifugation, the supernatant was adjusted to 500 mM NaCl. For immunoprecipitation, 200 μ l of cell lysate was diluted to 1 ml with 500 mM NaCl/50 mM Tris-HCl, pH 8.0/0.5% Nonidet P-40/0.1% NaDodSO₄ containing protease inhibitors as above. Five microliters of anti-human insulin monoclonal antibody 83-14 (20) (kindly provided by K. Siddle, University of Cambridge, U.K.) and 50 μ l of protein A-agarose (Pierce) were added. After 4 hr of incubation at 4°C, immunoprecipitates were sedimented and washed (21).

RESULTS

Isolation of Cell Lines Overexpressing Insulin Receptors. Thirty individual G418-resistant cell lines were isolated after co-transfection of NIH 3T3 cells with pBPVHIRA⁻ and pSV2neo. Of these cell lines, seven demonstrated [¹²⁵I-Tyr-A14]insulin binding (range 2.7–16.9% of input cpm) that was at least 4-fold that of G418-resistant cells obtained after transfection with pSV2neo alone [$0.5 \pm 0.13\%$ (mean $\pm$ SEM, $n = 5$)]. As the expression of the human insulin receptor cDNA is under the control of the mouse metallothionein promoter in pBPVHIRA⁻, we anticipated that it would be induced by exposure to heavy metals. However, after overnight culture in the presence of 4 μ M cadmium, no further insulin receptor-expressing cell lines could be detected. Furthermore, a maximum increase of 50% in insulin binding could be demonstrated in four of these seven cell lines. In NIH 3T3 HIR2.1 and -3.5, the lines showing the highest levels of insulin binding, cadmium had no demonstrable effect. NIH 3T3 HIR3.5, the cell line expressing the human insulin receptor with the greatest efficiency, was selected for further study.

Insulin Binding to NIH 3T3 HIR3.5 Cells. To characterize insulin binding to NIH 3T3 HIR3.5 cells, competitive binding studies were performed. The results of such an experiment are shown as a displacement curve in Fig. 2A. The concentration of unlabeled insulin required for half-maximal displacement of [¹²⁵I-Tyr-A14]insulin was ≈ 1 nM, similar to that observed for human cells (23). When these data were transformed by the method of Scatchard (22), a typical curvilinear plot was obtained (Fig. 2B). The number of receptors per cell estimated from the abscissa intercept of this plot is 6×10^6 . Computer analysis of these data, by a modification of the "ligand" program (24) (kindly donated by B. Ginsberg, University of Iowa), indicated that there are 3×10^6 high-affinity receptors ($K_d = 1.09 \times 10^{-9}$ M) and 5×10^6 low-affinity receptors ($K_d = 10^{-7}$ M) per cell. A further 3-fold increase in insulin receptor concentration could be induced by culturing the cells for 18 hr in the presence of 5 mM sodium butyrate (data not shown).

Human Insulin Receptor Structure in NIH 3T3 HIR3.5 Cells. To determine the subunit structure of the human insulin receptors expressed in the NIH 3T3 HIR3.5 cells, the cells were biosynthetically labeled with [³⁵S]methionine and cleared detergent lysates were immunoprecipitated with the species-specific anti-human insulin receptor monoclonal antibody 83-14 (ref. 24 and J.W., unpublished data). Lysates of [³⁵S]methionine-labeled IM-9 lymphocytes (a human cell line) and untransformed NIH 3T3 cells were also immunoprecipitated, by 83-14 in the case of the IM-9 cells and by a rabbit polyclonal anti-insulin receptor antibody (20) in

FIG. 1. Expression vector pBPVHIRA⁻. The vector pBPVMT-HXho contains a 5.5-kb subgenomic fragment of BPV (hatched box), the mouse metallothionein-I (MT-I) gene (solid line), and the prokaryotic pML sequences (open boxes). A human kidney insulin receptor cDNA (HIRA⁻) was subcloned into the single *Xho I* site. The presumed structure of the MT-I/HIRA⁻ chimeric spliced mRNA is shown above the plasmid circle.

FIG. 2. Insulin binding to NIH 3T3 HIR3.5 cells. Cells in 24-well dishes (2.4×10^4 per well) were incubated with [¹²⁵I]-Tyr-A14]insulin (30 pM) and the indicated concentrations of unlabeled insulin in a total volume of 200 μ l of Hanks' balanced salts solution supplemented with 50 mM Hepes (pH 7.6) and 10 mg of bovine serum albumin per ml for 16 hr at 4°C. Incubations were terminated and insulin specifically bound to the cells was determined. (A) Competitive binding curve, with results expressed as percent of input [¹²⁵I]-labeled insulin bound. (B) The same data transformed by the method of Scatchard (22).

the case of the NIH 3T3 cells. Three major labeled proteins were precipitated from the NIH 3T3 HIR3.5 cell lysate (Fig. 3). Rather surprisingly, the two bands corresponding to the α and β subunits of the insulin receptor had M_r values of 147,000 and 105,000, respectively (compared to M_r 138,000 and 95,000 for the IM-9 lymphocytes). However, these values are identical to the apparent molecular weights of the α and β subunits immunoprecipitated from the wild-type NIH 3T3 cells (Fig. 3). The band at M_r 115,000 observed with these cells probably represents a proteolytic degradation product of the α subunit (J.W., unpublished observations). The third protein immunoprecipitated from both transformed and wild-type NIH 3T3 cells is the insulin receptor precursor, of M_r 190,000, identical to that reported for IM-9 lymphocytes by Hedro *et al.* (21). Thus, the differences in size of the mature insulin receptor subunits between the NIH 3T3 HIR3.5 cells and the IM-9 lymphocytes are probably due to differences in carbohydrate composition.

To investigate this possibility, immunoprecipitates of lysates of [³⁵S]methionine-labeled NIH 3T3 HIR3.5 cells and IM-9 lymphocytes were subjected to N-glycanase (peptide-N glycosidase F, Genzyme, Norwalk, CT) digestion to cleave the N-linked oligosaccharides from the core protein. In both cell types, treatment of the insulin receptor with N-glycanase produced a decrease in size of both α and β subunits, resulting in a broad band at $M_r \approx 93,000$ (Fig. 4A). To

FIG. 3. Insulin receptor subunit structure. NIH 3T3 HIR3.5 cells (lane 1), IM-9 lymphocytes (lane 2), and untransformed NIH 3T3 cells (lane 3) were labeled overnight with [³⁵S]methionine (250 μ Ci/ml; 1 Ci = 37 GBq). Cleared detergent lysates of the cells were immunoprecipitated with anti-human insulin receptor monoclonal antibody 83-14 in the case of NIH 3T3 HIR3.5 and IM-9 cells and with a rabbit anti-insulin receptor polyclonal antibody in the case of the untransformed NIH 3T3 cells; it was necessary to concentrate the insulin receptor of untransformed NIH 3T3 cells by chromatography on lentil lectin agarose (Vector Laboratories, Burlingame, CA) (25) prior to immunoprecipitation. Immunoprecipitates were analyzed by NaDodSO₄/7.5% polyacrylamide gel electrophoresis and autoradiography. Apparent molecular weights ($M_r \times 10^{-3}$) of receptor subunits are given at left; values for standard proteins run in parallel are given at right.

demonstrate the effect of N-glycanase on α and β subunits individually, [¹²⁵I]-Tyr-B26]insulin was crosslinked with disuccinimidyl suberate (33) to the α subunits of insulin receptors of both cell types, detergent extracts were prepared, and immunoprecipitates were digested with the enzyme. Similarly, β subunits of wheat germ agglutinin-purified detergent extracts of both cell lines were labeled with [³²P]ATP in the presence of insulin, immunoprecipitated, and digested. N-glycanase decreased the apparent molecular weight of α subunits of the insulin receptors of both cell lines, giving a single band at M_r 100,000 (Fig. 4B). In contrast, N-glycanase treatment of the β subunits of both cell lines produced two bands at M_r 93,000 and 84,000 (Fig. 4C), suggesting that the β subunit may be more resistant to this enzyme than the α subunit. These data thus confirm that the increased size of the α and β subunits of the human insulin receptor expressed by NIH 3T3 HIR3.5 cells is due to differences in N-linked oligosaccharide composition, resulting from differences in posttranslational processing of the receptor in this cell type.

To determine whether these changes were due to an increase in sialic acid content or an increase in the complexity of the oligosaccharide side chains, immunoprecipitates of lysates of [³⁵S]methionine-labeled NIH 3T3 HIR3.5 cells and IM-9 lymphocytes were treated with *Vibrio cholerae* neuraminidase (Calbiochem) to remove sialic acid from the insulin receptor. This reduced the molecular weight of the NIH 3T3 HIR3.5 receptor α subunit to 138,000 and that of the IM-9 α subunit to 132,000 (Fig. 5). The molecular weight of the NIH 3T3 HIR3.5 β subunit was decreased to 100,000 and that of the IM-9 β subunit to 93,000. While this suggests that the sialic acid content of the insulin receptor subunits of the NIH 3T3 HIR3.5 cells is increased, the complexity of the oligosaccharide chains of the subunits must also be increased in order to account for the observed differences in size between the subunits of the two cell types.

Insulin Receptor Biosynthesis. As it has been suggested that different rates of transit of the glycoproteins to and through the Golgi apparatus can influence the nature of their oligosaccharide side chains (27), insulin receptor biosynthesis and

FIG. 4. N-linked oligosaccharides of insulin receptors. Insulin receptors of NIH 3T3 HIR3.5 cells and IM-9 cells were either labeled biosynthetically with [^{35}S]methionine (A) or affinity-labeled with [^{125}I -Tyr-B26]insulin (B) and detergent lysates were prepared. (C) Insulin receptors from both cell types were also partially purified by wheat germ agglutinin chromatography (25) and phosphorylated with [γ - ^{32}P]ATP in the presence of 0.1 μM insulin (26). Immunoprecipitates with monoclonal antibody 83-14 were prepared from each preparation and incubated overnight in the presence or absence of N-glycanase. Samples were analyzed by NaDodSO₄/7.5% polyacrylamide gel electrophoresis and autoradiography. Apparent molecular weights ($M_r \times 10^{-3}$) of receptor subunits are shown.

processing were studied by pulse-chase labeling the cells with [^{35}S]methionine. After a 15-min pulse, a faint band at M_r 190,000 was visible (Fig. 6). This band attained maximal intensity after 60 min of the chase and decreased in intensity thereafter. Concomitant with this decrease in intensity, the mature α and β subunits appeared, being first detectable at 2 hr and reaching maximal intensity at 7 hr. Densitometric analysis showed the half-life of the precursor to be 90 min. This pattern is very similar to that observed for IM-9 lymphocytes by Hedo *et al.* (21). Thus, differences in the kinetics of posttranslational processing of insulin receptor are unlikely to account for the changes in carbohydrate composition.

Glycogen Synthesis. To determine whether the insulin receptors expressed in the transfected cells were coupled to a metabolic response to insulin, the effects of insulin (0.1 nM) on glycogen synthesis were determined by quantitation of

D-[U- ^{14}C]glucose incorporation into glycogen in the presence or absence of insulin (Fig. 7). In the control, pSV2neo-transfected cells, insulin had no effect, even when concentration was increased to 10 nM (data not shown). In the NIH 3T3 HIR3.5 cells, the basal level of glycogen synthesis was increased 4- to 5-fold compared with the control cells, and insulin produced a further 50% increment in the rate of glycogen synthesis. This constitutive increase in the basal rate of glycogen synthesis is very similar to the increase in the basal rate of glucose uptake by CHO cells overexpressing insulin receptors, reported by Ellis *et al.* (8).

DISCUSSION

The results show that human insulin receptor cDNAs can be overexpressed in NIH 3T3 fibroblasts transfected with BPV vector; approximately 20% of the G418-resistant clones (7 out of 30) arising from a single cotransfection expressed detectable quantities of insulin receptor. Of these, two, NIH 3T3 HIR3.5 and NIH 3T3 HIR2.1 (data not shown) had 6×10^6 and 1.5×10^6 receptors per cell, respectively. The receptor concentration of NIH 3T3 HIR3.5 could be further increased to 2×10^7 per cell (i.e., 10 μg of receptor per 10^6 cells) by induction with sodium butyrate. Rather surprisingly, the sizes of the α and β subunits of the insulin receptors expressed in the NIH 3T3 cells were larger than those thought to be typical of the human insulin receptor, although they

FIG. 5. Neuraminidase treatment of insulin receptors. Cleared detergent lysates of NIH 3T3 HIR3.5 cell and IM-9 lymphocytes labeled overnight with [^{35}S]methionine were immunoprecipitated with monoclonal antibody 83-14. Immunoprecipitates were treated with 0.2 unit of neuraminidase from *Vibrio cholerae* for 30 min at 37°C and then electrophoresed in NaDodSO₄/7.5% polyacrylamide gels. Positions of standards ($M_r \times 10^{-3}$) run in parallel are indicated at left.

FIG. 6. Insulin receptor biosynthesis. Cells at 75% of confluence in 100-mm dishes were labeled for 15 min with [^{35}S]methionine (250 $\mu\text{Ci}/\text{ml}$) in 4 ml of methionine-deficient Dulbecco's modified Eagle's medium (DMEM) containing 5% dialyzed fetal bovine serum. To initiate the chase, labeling medium was removed, cells were washed twice with phosphate-buffered saline, and 4 ml of warmed DMEM/10% fetal bovine serum supplemented with 2 mM unlabeled methionine was added. At the chase time points (min) given above the lanes, cells were solubilized and immunoprecipitates were analyzed by electrophoresis and autoradiography.

FIG. 7. Glycogen synthesis in the absence (–) or presence (+) of 0.1 nM insulin. Glycogen synthesis in control cells transfected with pSV2neo only (NEO) and in NIH 3T3 HIR3.5 cells was determined by quantitation of D-[U-¹⁴C]glucose incorporation into glycogen by the method of Hofmann *et al.* (28) with the following minor modifications: cells were incubated with glucose-free medium for 18 hr prior to assay, and unlabeled glucose was omitted from the assay medium.

were the same as those of the subunits of the wild-type NIH 3T3 receptor. We have shown this to be due to differences in N-linked oligosaccharide content.

There have been reports of insulin receptors with atypical subunits (29–31), and a precedent exists for a human insulin receptor with α and β subunits of increased size in the receptor of the U937 monocyte cell line (31). The α and β subunits of the receptors of these cells were shown to have an increased sialic acid content but this did not completely account for the increased subunit size. Although the carbohydrate composition of these receptors was not investigated in detail, our findings with human insulin receptors expressed in NIH 3T3 cells suggest that changes in carbohydrate composition can entirely account for the increased subunit size of these receptors. Thus, while the processing of human insulin receptor mRNAs is complex (2, 3) and could potentially lead to variations in structure of the core protein by alternative splicing mechanisms, we propose that much of the variability of insulin receptor subunit size observed in human and animal cells arises from variation in the glycosylation machinery of the host cell.

Whether such variations in glycosylation are functionally significant remains to be determined. Certainly, the human insulin receptors expressed in NIH 3T3 cells appear to be grossly normal in terms of their insulin-binding function, ability to autophosphorylate, and stimulation of glycogen synthesis. Thus, overexpression of human insulin receptor cDNAs with BPV vectors in NIH 3T3 cells should serve as a suitable system for the investigation of insulin receptor function by site-directed mutagenesis.

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