Specific Glycosylation Site Mutations of the Insulin Receptor α Subunit Impair Intracellular Transport†

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Received February 22, 1993; Revised Manuscript Received April 13, 1993

ABSTRACT: The insulin receptor is a transmembrane protein found on multiple cell types. This receptor is synthesized as a 190-kDa proreceptor which is cleaved to produce mature α and β subunits. The proreceptor contains 18 potential sites for N-linked glycosylation: 14 on the α subunit and 4 on the β subunit. The codons for asparagine in the first four sites at the amino terminus of the α subunit were mutated to code for glutamine. This mutant receptor cDNA was stably transfected into NIH 3T3 cells. The insulin receptor produced in these cells remained in the proreceptor form; no mature α and β subunits were produced. The proreceptor was slightly smaller on SDS–PAGE gels than the wild-type proreceptor and contained four less oligosaccharide chains by tryptic peptide mapping. The carbohydrate chains on the mutant proreceptor remained endoglycosidase H sensitive. However, in the presence of brefeldin A, these oligosaccharide chains could be processed to endoglycosidase H resistant chains. By immunofluorescence, the mutant proreceptor was shown to be localized to the endoplasmic reticulum. No insulin receptors could be found on the cell-surface either with cell surface labeling with biotin or with ^{125}I-insulin binding. Thus, glycosylation of the first four N-linked glycosylation sites of the insulin receptor is necessary for the proper processing and intracellular transport of the receptor. This is in contrast to glycosylation at the four sites on the β subunit which appear not to be important for processing but necessary for signal transduction. Therefore, N-linked glycosylation of the insulin receptor at specific sites has multiple distinctive roles.

The insulin receptor, a membrane glycoprotein composed of two heterodimers linked by disulfide bonds (Jacobs & Cuatrecasas, 1981), contains covalently bound fatty acids (Hedo et al., 1987), N-linked oligosaccharides (Hedo et al., 1981; Van Obberghen et al., 1981), and O-linked oligosaccharides (Collier & Gorden, 1991; Herzberg et al., 1985). Previously we investigated the location and function of the O-linked oligosaccharides on the insulin receptor (Collier & Gorden, 1991), and here we address the function of specific N-linked oligosaccharides to the insulin receptor. On the basis of the cDNA sequence of the insulin receptor (Ullrich et al., 1985; Ebina et al., 1985), there are 14 potential sites for N-linked glycosylation on the α subunit, which is exclusively extracellular (Hedo & Simpson, 1984), and 4 potential sites on the extracellular portion of the β subunit. Asn16 and Asn397 in the α subunit and all of the potential sites in the β subunit are known to be glycosylated (Collier & Gorden, 1991; Ullrich et al., 1985; Ebina et al., 1985; Hayes et al., 1991). We have suggestive evidence that a majority of the potential glycosylation sites, if not all, are actually utilized (Collier & Gorden, 1991; unpublished observations). The receptor is made as a 190-kDa precursor containing only high-mannose oligosaccharides (Hedo et al., 1983). A majority of these high-mannose oligosaccharide chains undergo trimming and processing to complex chains with addition of *N*-acetylglucosamine, galactose, fucose, and sialic acid (Hedo et al., 1981, 1983; Hedo & Gorden, 1985). The precursor is cleaved at the Arg-Lys-Arg-Arg site to form the α (135 kDa) and β (95 kDa) subunits; two precursors are disulfide-linked before insertion in the membrane (Hedo & Gorden, 1985). The mature receptor contains both high-mannose oligosaccharides and processed complex carbohydrates (Hedo et al., 1983). Studies using inhibitors of various steps in the N-linked glycosylation path have suggested this process is important for the normal intracellular transport of the receptor. Recently, however, mutation of the four glycosylation sites on the β subunit was shown to have no effect on the processing of the insulin proreceptor (Leconte et al., 1992). Further, the interpretation of studies with glycosylation inhibitors is complicated by the fact that these agents are active on all glycoproteins, so that the effects on processing of the insulin receptor could be through an indirect effect of another protein. Equally, the effect of the inhibitors on processing could be due to inhibition of glycosylation at a subset of the N-linked sites. The inhibitor studies do not allow one to determine if specific sites of glycosylation play distinctive roles. To address the role of the amino-terminal glycosylation sites of the α subunit, we mutated the cDNA of the human insulin receptor so that the first four potential sites of N-linked glycosylation in the α subunit contain glutamine instead of asparagine. This change was selected as the most conservative amino acid change that would prevent glycosylation at these sites. This cDNA was then stably transfected into NIH 3T3 cells. The insulin receptor expressed in these cells is retained in the endoplasmic reticulum, not expressed on the cell surface, and not processed past the proreceptor form. The other oligosaccharides appear to be added appropriately to the receptor, but remain endoglycosidase H sensitive.

EXPERIMENTAL PROCEDURES

Materials. [^{35}S]dATP (1000–1500 Ci/mmol), [2-^{3}H]-mannose (30 Ci/mmol), [^{35}S]methionine (1000 Ci/mmol), and EN^{3}HANCE were purchased from Dupont—New En-

† L.B. was supported by the National Institutes of Health—Howard Hughes Medical Institute Research Scholars Program.

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0006-2960/93/0432-7818$04.00/0

gland Nuclear (Boston, MA). DMEM[1] medium was from Biofluids (Rockville, MD). TPCK-trypsin was from Worthington Biochemicals (Freehold, NJ). Brefeldin A was from Epicentre Technologies (Madison, WI). HPLC-grade solvents were from Burdick and Jackson (Muskegon, MI). Sulfosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC-biotin) and spectra-grade TFA (trifluoroacetic acid) were from Pierce (Rockford, IL). Sequenase brand of T7 DNA polymerase was from USB (Cleveland, OH). Oligonucleotides were made on an ABI 394 DNA/RNA synthesizer. Restriction enzymes, T4 ligase, and calf alkaline phosphatase were from Boehringer Mannheim (Indianapolis, IN). ATP, proteinase K, RNase A, ampicillin, and lysozyme were from Sigma (St. Louis, MO). ^{125}I-Insulin (receptor grade, 2000 Ci/nmol), ECL Western blotting detection reagents, and horseradish peroxidase conjugated to streptavidin were from Amersham (Arlington Heights, IL). Goat anti-rabbit IgG coupled to horseradish peroxidase was from Bio-Rad (Melville, NY). G-418 (geneticin), lipofectin, and protein A–agarose were from GIBCO BRL (Gaithersburg, MD). RNAzol was from Tel-Test (Friendswood, TX).

Mutant Construction. The wild-type insulin receptor cDNA with an SV40 polyadenylation site was previously constructed in PGEM 4Z (Kadowaki et al., 1988). The N-linked glycosylation sites were mutated in groups of two (Asn16 and Asn25, and Asn78 and Asn111). Mutations in the first two sites (CAG for AAC and AAT) were produced in an *Eco*RV–*Xho*I segment of the insulin receptor by use of overlapping PCR with mutant primers (Higuchi et al., 1988; Kadowaki et al., 1989). The second two sites (CAG for AAC) were produced by the same method in an *Xho*I–*Kpn*I segment of the receptor. These pieces were ligated into separate cloning vectors. These plasmids were amplified in bacteria, the plasmid DNA was isolated, and the area of the insert pieces was sequenced using different primers than those used in the PCR. Clones with the site-directed mutations and normal sequence in the rest of the insert were selected. The plasmid DNA from these clones was cut with the appropriate enzymes, and the insert pieces were gel-purified. A triple ligation was performed to substitute the mutant pieces into the normal *Eco*RV–*Kpn*I site in a cDNA encoding the normal full-length insulin receptor. A clone was selected; the nucleotide sequence was confirmed in the ligated region to contain the mutations and otherwise normal sequence. Then the mutated insulin receptor cDNA was ligated into a bovine papilloma virus based expression vector in which insulin receptor cDNA expression is driven by the murine metallothionein promoter (Kadowaki et al., 1988). NIH 3T3 cells were transfected using lipofectin containing the expression vector and a plasmid containing neomycin resistance (pRSVNeo). After selection by culturing cells in the presence of the antibiotic G418 (600 μg/mL; Gibco), stable transfectants were isolated, cloned, and cultivated in dishes. The expression of the mutant DNA in these cells was checked by isolating RNA using RNAzol, and sequencing of the region of interest after reverse transcriptase and PCR amplification.

Cells. NIH 3T3 fibroblasts transfected with human insulin receptor cDNA, either mutant or wild type, were grown in DMEM with high glucose, 10% fetal calf serum, and G418 (600 μg/mL). When used for an experiment, the cells were grown in the above media until just prior to confluence.

Labeling of Cells. A total of 10 150-mm plates were used for each experiment with the NIH 3T3 fibroblasts. Cells were incubated in methionine-free or glucose-free media with dialyzed fetal calf serum for at least 1 h. The cells are then pulsed with the same media containing 5 mCi of [^{35}S]methionine or 25 mCi of [^{3}H]mannose for 15–45 min. After the cells were washed 3 times with normal media or media containing 2 mM mannose, the cells were then chased in this same media for 0–20 h depending on the experiment. For those experiments with brefeldin A, it was present throughout the preincubation, pulse, and chase periods. The chase was stopped by washing the cells 3 times with cold PBS. The cells were solubilized as previously described (Arakaki et al., 1987). Briefly, the cells were scraped from the plates into solubilization buffer [1.5% Triton X-100 in 50 mM Hepes/150 mM NaCl, pH 7.6, with 2 mM PMFS and aprotinin (15 trypsin inhibitor units/mL)] and then stirred for 30 min at 4 °C. The particulate material was sedimented by centrifuging at 200000*g* for 45 min. The supernatant was then preadsorbed with protein A–sepharose for 1 h, after which the insulin receptor was immunoprecipitated overnight with anti-peptide antibody 46B (1:100 dilution). This is a polyclonal antiserum made to a cytoplasmic peptide of the β subunit of the insulin receptor [S. Fuchs and S. I Taylor, similar to Ab 46 in Cama et al., (1988)]. The receptor was precipitated with protein A and boiled in sample buffer (2% SDS, 0.1 mM dithiothreitol, 0.002% bromphenol blue, 10% glycerol, and 10 mM phosphate) prior to separation of the proreceptor and α and β subunits by SDS–polyacrylamide gel electrophoresis. For those experiments with BFA, the samples were eluted from the protein A in 1% SDS, 10 mM DTT, and 10 mM phosphate buffer, pH 7.0, with boiling. The samples were concentrated and resuspended in 150 mM citrate, pH 5.5, and treated with or without endoglycosidase H overnight at 37 °C. The sample was precipitated with 10% trichloroacetic acid and the precipitate washed with ether/ethanol before resuspension in sample buffer. Electrophoretic separation and fluorography of the dried gels were performed as described previously (Arakaki et al., 1987). Tryptic peptide mapping was performed as previously described (Collier & Gorden, 1991).

Biotinylation of Cell Surface Receptors. Confluent monolayers of NIH 3T3 fibroblasts were biotinylated as described by Musil and Goodenough (1991). Briefly, 10-cm dishes of cells were washed with PBS containing 0.1 mM $CaCl_2$ and 1.0 mM $MgCl_2$ (PBS/Ca/Mg) 3 times at 4 °C and then incubated with gentle agitation for 30 min at 4 °C with 10 mL of 0.5 mg/mL NHS-LC-biotin in PBS/Ca/Mg. The reaction was quenched by washing the cells 3 times with PBS/Ca/Mg containing 15 mM glycine, with a 10-min incubation between the second and the third wash. The cells were then solubilized and immunoprecipitated as previously described except that anti-peptide antibody 50 (Cama et al., 1988) was used. This is an antibody to a different peptide also in the cytoplasmic tail of the β subunit of the human insulin receptor. Proteins were then separated using SDS–PAGE and transferred to nitrocellulose filters. The filters were blocked for 2 h in PBS containing 10% dry milk and 0.1% Tween-20, and incubated for 1 h with horseradish peroxidase conjugated streptavidin (1:500) in PBS/Tween-20. Filters were then washed extensively in PBS/Tween-20 and the proteins visualized utilizing the ECL detection system. As a control,

[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; FITC, fluorescein isothiocyanate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; BFA, brefeldin A; BiP, immunoglobulin heavy chain-binding protein.

cells from similar dishes were solubilized, separated without immunoprecipitation on SDS–PAGE, and, after transfer to nitrocellulose, probed with anti-peptide antibody 50. For the anti-peptide antibody blotting, the filters were soaked for 2 h at room temperature in PBS containing non-fat dry milk (100 mg/mL) and then incubated overnight at 4 °C in PBS with 0.1% Tween-20 and 0.4% bovine serum albumin and rabbit anti-insulin receptor antibody 50 (1:2000). After extensive washing with PBS/Tween-20, the filters were incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (1:10000) for 30 min at room temperature. Detection of the proteins was by use of the ECL method.

Insulin Binding. Cells were plated in 6-well plates and grown until confluence. The plates were washed once with PBS prior to incubation with ^{125}I-insulin and varying concentrations of unlabeled insulin for 75 min at 15 °C. The cells were then washed twice with cold PBS, solubilized in 1 M NaOH, and counted in a γ counter. Nonspecific binding was subtracted from each sample.

Immunofluorescence. To localize the insulin receptor, NIH 3T3 cells grown on coverslips were fixed and permeabilized by acetone at –80 °C. Briefly, cells were washed 5 times in 0.1 M PBS, pH 7.4 at 4 °C, covered with acetone at –80 °C, and incubated at –20 °C for 3 min; then the acetone was removed, the cells were dried for 5 min, and PBS at 4 °C was added. Cells were washed with PBS at 22 °C before incubation for 2 hours at 22 °C with the PBS containing the monoclonal anti-insulin receptor antibody (mAb 83-14) provided by D. K. Siddle (Cambridge, England) at 10 μg/mL. Cells were washed in PBS prior to incubation with FITC-conjugated rabbit anti-mouse IgG at a dilution of 1:200 for 1 h at 22 °C, rinsed in PBS, and counterstained with Evans blue before examination in a Zeiss Axiophot fluorescence microscope. Control experiments included incubation in the presence of second antibody alone or incubation in the presence of an irrelevant first antibody. The distribution of the fluorescent reaction obtained with mAb 83–14 was also compared to that obtained with an antibody directed against an endoplasmic reticulum protein (BiP) provided by Dr. S. Fuller (EMBL, Heidelberg, Germany) or against the Golgi proteins provided by Dr. D. Louvard (Institut Pasteur, Paris, France).

RESULTS

The cDNA for the insulin receptor with the first four potential glycosylation site Asn codons mutated to codons for Gln was transfected into NIH 3T3 fibroblasts along with the RSVNeo plasmid so that selection based on G418 resistance could be used. Several clones were selected based on G418 resistance and these cells labeled biosynthetically overnight with [^{35}S]methionine. The cells were solubilized; the insulin receptors were immunoprecipitated with an anti-peptide antibody to a cytoplasmic portion of the insulin receptor and separated on SDS–PAGE. As shown in Figure 1, at least three of the clones express increased amounts of proreceptor, which is smaller than the wild-type insulin proreceptor. The mutant cell lines do not show processing of the proreceptor to the mature α and β subunits.

A time course of the biosynthetically labeled receptor is shown in Figure 2. The cells were labeled for 1 h with [^{3}H]mannose and then chased in unlabeled media supplemented with 2 mM mannose for the indicated number of hours. As shown in Figure 2A, the wild-type receptor is clearly processed to produce the mature α and β subunits with a peak of mature subunits at 6 h of chase. As is seen in Figure 2B, the mutant insulin receptor shows a proreceptor band that is

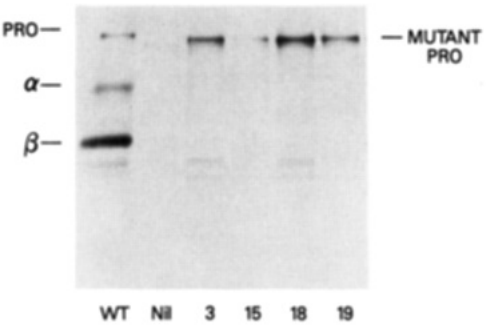

FIGURE 1: Screening of clones of NIH 3T3 cells transfected with the mutant DNA. Cells were labeled overnight with [^{35}S]methionine. The cells were solubilized; insulin receptors were immunoprecipitated with an anti-peptide antibody and separated on a 7.5% polyacrylamide gel. PRO, proreceptor; α, alpha subunit; β, β subunit; WT, cells transfected with the wild-type insulin receptor cDNA; Nil, non-transfected cells; 3, 15, 18, and 19, four separate clones of cells transfected with the mutant cDNA (Q-16, Q-25, Q-78, Q-111).

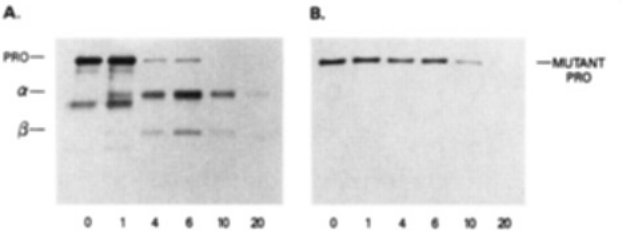

FIGURE 2: Time course of biosynthesis of the insulin receptor. (A) Cells transfected with the wild-type insulin receptor cDNA were labeled with [^{35}S]methionine for 30 min and then chased with unlabeled media supplemented with 2 mM methionine for the indicated number of hours. The cells were then solubilized, and insulin receptors were immunoprecipitated, and proteins were separated on SDS–PAGE. (B) Same as (A) except cells transfected with the mutant insulin receptor cDNA were used.

slightly smaller than that of the wild type. However, this proreceptor is not further processed to produce mature α and β subunits. The proreceptor is long-lived with a half-life of approximately 8 h, while the half-life of the wild-type proreceptor is approximately 1 h. The mutant proreceptor is slightly smaller than the wild-type receptor as would be expected if it contained four less oligosaccharide chains. Comparison of the peak maps of [^{3}H]mannose-labeled tryptic peptides made from the wild-type proreceptor and the mutant proreceptor separated on HPLC showed the loss of four different peaks. This is consistent with the loss of the four glycosylated peptides in the mutant proreceptor (data not shown).

In wild-type cells, the proreceptor is not found on the cell surface (Forsayeth et al., 1986). Since the mutant proreceptor is not processed to the mature α and β subunits, it would be expected that cell-surface insulin binding would be markedly diminished. When mutant cells were incubated with ^{125}I-insulin, binding was similar to the background levels found in untransfected cells (data not shown). However, the lack of binding could be due to the lack of receptor molecules on the cell surface or to the inability of mutant receptors to bind insulin. To assess whether mutant insulin receptor molecules are found on the cell surface, cell-surface proteins were biotinylated on lysines with NHS-LC-biotin. The insulin receptor was immunoprecipitated, separated on SDS–PAGE, and transferred to nitrocellulose, and those receptors from the cell surface were detected with strepavidin. In Figure 3, in cells expressing the wild-type receptor, α and β subunits can be detected, whereas in the cells expressing the mutant receptor, there are no insulin receptors on the cell surface. A

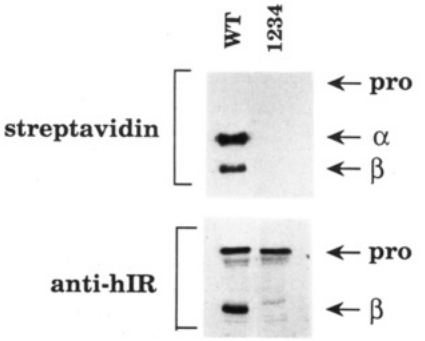

FIGURE 3: Biotinylation of cell-surface receptors. Cells were labeled with biotin and solubilized, insulin receptors were immunoprecipitated, and the proteins were separated on SDS–PAGE. After transfer of the proteins to nitrocellulose, the blot was probed with horseradish peroxidase–streptavidin and detected using the ECL Western blot analysis system. For the anti-hIR blot, similar dishes of cells were solubilized, and proteins were separated on SDS–PAGE, transferred to nitrocellulose, and probed with the anti-peptide antibody 50. Detection was by the horseradish peroxidase labeled anti-rabbit IgG and ECL Western blot analysis system. WT, wild type; 1234, mutant.

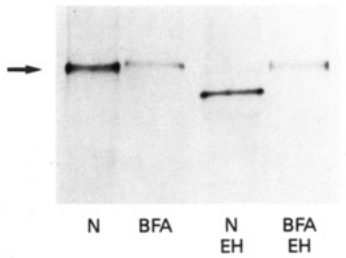

FIGURE 4: Analysis of the carbohydrates of the insulin receptors of cells transfected with the mutant cDNA grown in normal media or media supplemented with brefeldin A. Cells were treated or untreated with brefeldin A for 1 h and then labeled with [^{35}S]methionine for 1 h in the presence or absence of BFA. The cells were solubilized, and the insulin receptors were immunoprecipitated and then treated or not treated with endoglycosidase H before separation on a gel. N, normal media; BFA, media supplemented with BFA; EH, endoglycosidase H.

blot of proteins from similarly solubilized mutant cells with an antibody to a peptide from the human insulin receptor (Ab 50) shows that the cells did express the proreceptor. This suggests that the receptor is retained in the cell and is not transported to the cell surface.

The oligosaccharides on the mutant insulin proreceptor are all endoglycosidase H sensitive (Figure 4) as are the oligosaccharides on the wild-type insulin proreceptor (Collier & Gorden, 1991; data not shown). This is consistent with the retention of the mutant proreceptor in the endoplasmic reticulum. Another possibility is that the lack of the oligosaccharides on these four sites somehow interferes with the proper folding of the receptor such that it cannot be glycosylated even if the Golgi enzymes are available in the same compartment. To address this question, mutant cells were grown in brefeldin A, a compound which allows mixing of the Golgi and the endoplasmic reticulum compartments of the cell (Chege & Pfeffer, 1990). The mutant receptors would thus be allowed access to the Golgi compartment glycosylation enzymes. As shown in Figure 4, mutant cells grown in normal media produce proreceptors with endoglycosidase H sensitive oligosaccharides. However, mutant cells grown in the presence of brefeldin A produce proreceptors with oligosaccharides which are endoglycosidase H resistant. Thus, other oligosaccharides present at the nonmutated sites in the mutant proreceptor can be processed from high-mannose to complex

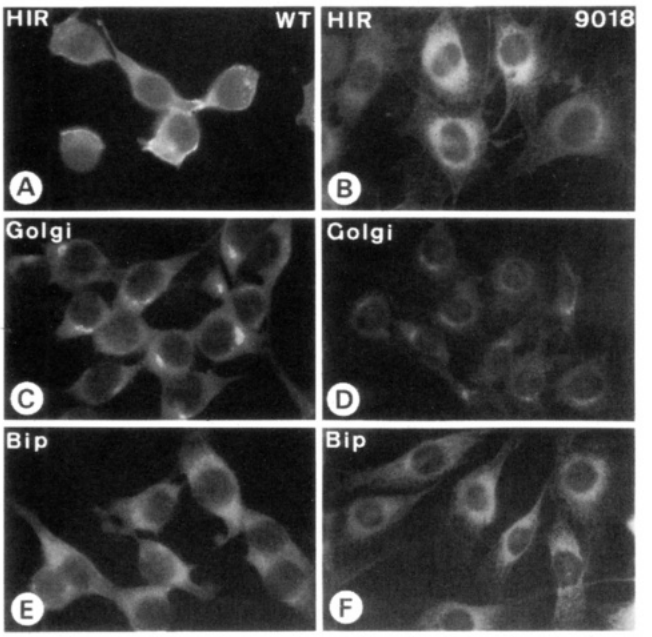

FIGURE 5: Comparison of the immunofluorescence staining obtained with anti-insulin receptor antibody (A and B), anti-Golgi antibody (C and D), and anti-BiP antibody (E and F) on fixed and permeabilized whole NIH 3T3 cells. Cells on the left (WT) (A, C, and E) expressed wild-type insulin receptors while cells on the right (9018) (B, D, and F) expressed the mutant insulin receptors. Magnification, 282×.

forms by the Golgi enzymes. Since in normal media the mutant proreceptor is not processed, it appears these mutant proreceptors are retained in the endoplasmic reticulum. Therefore, transport of the mutant receptors out of the endoplasmic reticulum to the Golgi compartment, or from the intermediate compartment into the trans-Golgi, is blocked.

To further localize the mutant receptors, the insulin receptor was examined by immunofluorescence. The cells were grown on slides and then made permeable with acetone prior to incubation with anti-insulin receptor antibody, anti-BIP antibody (endoplasmic reticulum), or anti-Golgi antibodies. As seen in Figure 5A, the wild-type insulin receptors are mostly associated with the cell surface. The localization of the Golgi complex and endoplasmic reticulum in these same cells is illustrated in Figure 5C,E, respectively. In cells expressing the mutant insulin receptors (Figure 5B,D,F), the distribution pattern of the receptors is clearly different from that observed in cells expressing the wild-type insulin receptors. In this case, the receptor distribution fits with the distribution of anti-BiP antibody localizing to the endoplasmic reticulum. This suggests that glycosylation of the insulin receptor near the N-terminus is somehow necessary for the correct intracellular transport of the protein through the endoplasmic reticulum to the Golgi where cleavage and further processing of the high-mannose oligosaccharides to complex forms occur.

DISCUSSION

The function of the N-linked oligosaccharides of several other cell-surface glycoproteins has previously been investigated by using site-directed mutagenesis of the sites for this glycosylation. The G protein of vesicular stomatis virus has been extensively investigated. One or the other of the two N-linked oligosaccharides is necessary and sufficient for the cell-surface expression. When both glycosylation sites are eliminated, the protein does not reach the cell surface (Machamer et al., 1985). Interestingly, the addition of new glycosylation sites to this mutant allowed cell-surface ex-

pression (Machamer & Rose, 1988). By immunofluorescence, the nonglycosylated VSV-G protein was retained in the Golgi compartment (Machamer et al., 1985). The CD4 protein, a T cell surface glycoprotein which is the receptor for the human immunodeficiency virus, contains two N-linked glycosylation sites. Glycosylation of either of these sites is also necessary and sufficient for the cell-surface expression of this protein (Tifft et al., 1992). In this case, the nonglycosylated protein is also retained intracellularly, but probably in the endoplasmic reticulum (Tifft et al., 1992). Thus, glycosylation is important for the cell-surface expression of these proteins, but the specific site may not be important.

In the hemagglutinin–neuraminidase protein of simian virus 5, another membrane glycoprotein, mutation of the four sites of N-linked glycosylation leads to impaired folding and intracellular transport of the protein. The severity of the defect in cell-surface expression depends on the number and the site of the mutations (Ng et al., 1990). Another protein where the specific site of glycosylation is important for cell-surface expression is the transferrin receptor. When all three N-linked glycosylation sites are mutated to produce a nonglycosylated receptor, the protein shows reduced cell-surface expression (Williams & Enns, 1991). Mutation of only the site nearest the transmembrane region results in retention of the receptor in the endoplasmic reticulum (Hoe & Hunt, 1992). The β-adrenergic receptor shows no loss of functional activity in the nonglycosylated state; however, the number of receptors on the cell surface is decreased by approximately 50% (Rands et al., 1990). By contrast, the muscarinic receptor shows very little change in function and intracellular transport when it is not glycosylated (Van Koppen & Nathanson, 1990).

Studies using inhibitors of glycosylation had suggested that glycosylation of the insulin receptor may be important. In cells treated with tunicamycin, which inhibits the cotranslational addition of the core oligosaccharide chain by interfering with the dolichol phosphate donor reaction, a 160–180-kDa precursor is produced (Goldstein & Kahn, 1988; Forsayeth et al., 1986; Reed et al., 1981; Ronnett et al., 1984; Ronnett & Lane, 1981). In these cells, there is no binding of insulin to the cell surface (Forsayeth et al., 1986; Rosen et al., 1979). In addition, cell-surface insulin binding is deceased by 40% in IM-9 lymphocytes treated with monensin, a carboxylic ionophore that interferes with glycosylation and intracellular transport of newly synthesized membrane proteins (Jacobs et al., 1983). Castanospermine and 1-deoxynojirimycin, inhibitors of the glucosidases that remove the glucoses from the core oligosaccharide as the first step in the processing of the high-mannose chains to complex chains, also decrease insulin binding on the cell surface by 50% (Arakaki et al., 1987). In these cells, a 205-kDa proreceptor was biosynthesized and slowly processed to normal-sized α and β subunits (135 and 95 kDa) (Arakaki et al., 1987). These data suggested that N-linked glycosylation is important for binding of insulin to the cell surface. None of these studies showed that the proreceptor was retained in the endoplasmic reticulum.

The insulin receptor is the only membrane receptor with relatively large numbers of glycosylation sites in which site-directed mutagenesis of these sites has been investigated. The present studies are the first to show specifically by site-directed mutagenesis the essential role of N-glycosylation on insulin receptor processing. This effect seems to be specific for the α subunit. Recently, all four of the N-linked glycosylation sites of the β subunit of the insulin receptor were mutated to prevent glycosylation. This mutant receptor had impaired biological activity, but intracellular transport of the receptor appeared normal (Leconte et al., 1992). Specific N-linked oligosaccharides of the insulin receptor can play different and distinctive roles in the receptor; those at the amino terminus of the α subunit are important for intracellular transport and those on the β subunit for the biological activity.

As always with mutagenesis experiments, we cannot exclude the possibility that the effect on intracellular transport is a result of the amino acid change rather than the lack of an oligosaccharide at these sites. However, in the context of the previous glycosylation inhibitor studies, it is likely that the effect on transport is due to the glycosylation defect.

The mechanism of the effect on intracellular transport is not clear. Since the first four sites of N-linked glycosylation in the proreceptor are mutated and these are the first sites to be glycosylated as the receptor is translated, it is interesting to speculate that these oligosaccharides are important in the initial proper folding of the receptor for further transport. The insulin receptors retained in the endoplasmic reticulum do not appear to be degraded through the recently described rapid endoplasmic reticulum degradation pathway (Klausner & Sita, 1990).

ACKNOWLEDGMENT

We gratefully acknowledge the contribution of the hIR cDNA by Axel Ullrich. We thank Rachel Myerowitz for critical reading of the manuscript.

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