Increased levels of insulin and insulin-like growth factor-1 hybrid receptors and decreased glycosylation of the insulin receptor α - and β -subunits in scrapie-infected neuroblastoma N2a cells

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We have previously shown that ScN2a cells (scrapie-infected neuroblastoma N2a cells) express 2-fold- and 4-fold-increased levels of IR (insulin receptor) and IGF-1R (insulin-like growth factor-1 receptor) respectively. In addition, the IR α - and β -subunits are aberrantly processed, with apparent molecular masses of 128 and 85 kDa respectively, as compared with 136 and 95 kDa in uninfected N2a cells. Despite the 2-fold increase in IR protein, the number of ¹²⁵I-insulin-binding sites was slightly decreased in ScN2a cells [Östlund, Lindegren, Pettersson and Bedecs (2001) Brain Res. 97, 161–170]. In order to determine the cellular localization of IR in ScN2a cells, surface biotinylation was performed, showing a correct IR trafficking and localization to the cell surface. The present study shows for the first time that neuroblastoma N2a cells express significant levels of IR–IGF-1R hybrid receptors, and in ScN2a cells the number of hybrid receptors was 2-fold higher than that found in N2a cells, potentially explaining the apparent loss of insulin-binding sites due to a lower affinity for insulin compared with the homotypic IR. Furthermore, the

decreased molecular mass of IR subunits in ScN2a cells is not caused by altered phosphorylation or proteolytic processing, but rather by altered glycosylation. Enzymic deglycosylation of immunoprecipitated IR from N2a and ScN2a cells with endoglycosidase H, peptide N-glycosidase F and neuraminidase all resulted in subunits with increased electrophoretic mobility; however, the 8–10 kDa shift remained. Combined enzymic or chemical deglycosylation using anhydrous trifluoromethane sulphonic acid treatment ultimately showed that the IR α - and β -subunits from ScN2a cells are aberrantly glycosylated. The increased formation of IR–IGF-1R hybrids in ScN2a cells may be part of a neuroprotective response to prion infection. The degree and functional significance of aberrantly glycosylated proteins in ScN2a cells remain to be determined.

Key words: glycosylation, hybrid receptor, insulin-like growth factor 1 receptor (IGF-1R), insulin receptor, prion, neuroblastoma, scrapie.

INTRODUCTION

In the central nervous system, insulin acts as an important neurotrophic factor, promoting cell growth and differentiation of neuronal cells during brain development [1,2]. In the brain, IR (insulin receptor) expression is developmentally regulated, but the IR continues to be expressed throughout the adult brain, with particularly high concentrations in the olfactory bulb, cerebral and cerebellar cortex and hippocampus (reviewed in [3]). The IR has been demonstrated to modulate synaptic activity both pre- and post-synaptically, and numerous studies have suggested that insulin is critical for normal brain function, including spatial and emotional learning and memory [4,5]. *In vitro*, insulin induces neuroblastoma cells (including N2a cells) to proliferate, and protects various neuronal cells from apoptosis [6,7].

The IR is closely related to the IGF-1R (insulin-like growth factor-1 receptor), with both belonging to the family of receptor tyrosine kinases. Both are tetrameric receptors composed of two α - and two β -subunits, and are each the product of a single gene (with that for IR located on chromosome 19, and that for IGF-1R on chromosome 15 in humans). The α -subunits contain the ligand-binding domains, and the β -subunits contain the transmembrane and protein tyrosine kinase domains [8,9].

The IR gene encodes a 190 kDa precursor that undergoes a number of processing steps. After being transported from the endoplasmic reticulum to the Golgi apparatus, the single glycosylated precursor is glycosylated further to a 210 kDa intermediate. Subsequently, the precursor is cleaved into the α - and β -subunits, both of which contain N-linked oligosaccharide chains and covalently linked fatty acids [10]. In addition, the β -subunit also contains O-linked oligosaccharides [11]. Immediately before insertion into the plasma membrane, capping of the α - and β -subunits by sialic acid occurs, giving rise to the mature 135 kDa α - and 95 kDa β -subunits, resulting in a partially endo H (endoglycosidase H)-resistant and neuraminidase-sensitive mature IR [10]. IR, and also IGF-1R, found in the brain and peripheral neurons differ from their corresponding receptors in peripheral tissues (liver, muscle and adipocytes) both structurally and biochemically, e.g. both the IR α - and β -subunits exhibit a lower molecular mass on SDS/PAGE. This is believed to be caused primarily by alterations in glycosylation, well illustrated by the insensitivity of brain IR to neuraminidase treatment, whereas the peripheral IR is neuraminidase-sensitive [12].

The central pathogenic event in prion diseases, e.g. Creutzfeldt–Jakob disease in humans and scrapie in sheep, is a post-translational conversion of a normal host-encoded glycoprotein, the

Abbreviations used: biotin-X-NHS, biotinamidocaproate *N*-hydroxysuccinimide ester; CIAP, calf-intestinal alkaline phosphatase; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; endo H, endoglycosidase H; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; IR, insulin receptor; IGF-1(R), insulin-like growth factor-1 (receptor); PAP, potato acid phosphatase; PNGase F, peptide N-glycosidase F; PrP^C, cellular prion protein; PrP^{Sc}, misfolded pathogenic isoform of the prion protein; RT-PCR, reverse transcriptase-PCR; ScN2a cells, scrapie-infected neuroblastoma N2a cells; ScN1E-115 cells, scrapie-infected N1E-115 cells; TFMSA, trifluoromethane sulphonic acid; WGA, wheat-germ agglutinin.

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PrP^C (cellular prion protein), into an abnormal, misfolded pathogenic isoform, designated PrP^{Sc}. This conversion appears to involve only a conformational change, and renders PrP^{Sc} insoluble in non-ionic detergents and partially resistant to proteolytic degradation [13]. Prion diseases are characterized by the accumulation of PrP^{Sc}, astrogliosis and vacuolization of nerve-cell processes, resulting in the typical spongiform neurodegeneration. PrP^C, which is mainly expressed in the brain, is a glycosyl-phosphatidylinositol-anchored protein, localized to sphingolipid- and cholesterol-rich membrane domains or 'lipid rafts' [14]. The PrP^{Sc} isoform is also found in these membrane domains [14].

We have previously studied how scrapie infection may affect IR and IGF-1R expression and function in scrapie-infected neuroblastoma cell lines [15,16]. Those studies showed that scrapie infection induces a 2- and 4-fold increase in IR and IGF-1R protein levels respectively, without a corresponding increase in specific ¹²⁵I-insulin and ¹²⁵I-IGF-1 binding sites or cell growth in insulin- or IGF-1-containing medium. In contrast with the elevated IGF-1R expression in ScN2a cells (scrapie-infected neuroblastoma N2a cells), receptor binding studies revealed an 80 % decrease in specific ¹²⁵I-IGF-1-binding sites. This decrease in IGF-1R-binding sites was shown to be caused by a 7-fold decrease in IGF-1R affinity. A similar decrease (65 %) in specific ¹²⁵I-insulin binding was found in ScN2a cells when the amount of ¹²⁵I-insulin binding sites was related to the increase in immunoreactive IR; however, in the case of the IR, the receptor binding affinity was unchanged.

The significant decrease in ¹²⁵I-insulin-binding sites in ScN2a cells, despite the unchanged affinity, indicated the presence of two populations of IRs: one receptor population that is normally expressed, processed and functional, as determined by an unchanged receptor binding affinity, and another receptor population that does not recognize insulin, but is detected by the anti-IR β antibody. In addition, the IR β -chain in ScN2a cells exhibited an increased electrophoretic mobility, with an apparent molecular mass of approx. 85 kDa, compared with 95 kDa in N2a cells [15]. These findings indicate a scrapie-induced dysregulation of the post-translational processing of the IR, including protein folding, glycosylation and trafficking. In this study we have characterized further the localization and altered post-translational processing of the atypical IR subunits in ScN2a cells, with the aim of identifying cellular changes that could contribute to prion-induced neurodegeneration.

MATERIAL AND METHODS

Materials

Anti-phosphotyrosine (4G10) antibody was purchased from Upstate Biotechnology, Inc.; anti-IR antibodies directed against the α - (sc-710) and β -subunit (sc-711) and anti-IGF-1R β (sc-713) were from Santa Cruz Biotechnology, Inc. Biotin-NHS-X (biotinamidocaproate *N*-hydroxysuccinimide ester) was from Calbiochem (cat. no. 203188). Recombinant anti-mouse PrP antibody D13 was kindly provided by Professor Stanley B. Prusiner at the University of California San Francisco, CA, U.S.A. Secondary HRP (horseradish peroxidase)-conjugated goat anti-human antibody was from ICN Biomedicals, Inc. HRP-conjugated anti-mouse IgG, anti-rabbit IgG and streptavidin were purchased from Amersham Biosciences, as well as the WGA (wheat-germ agglutinin) lectin-Sepharose beads. ¹²⁵I-insulin was from NEN Life Science Products Inc. (Brussels, Belgium). The IR-A and IR-B PCR primers were kindly given by Dr Ingo Leibiger at the Karolinska Institute. CIAP (calf-intestinal alkaline phosphatase), PNGase F (peptide N-glycosidase F), endo-H,

O-glycanase and neuraminidase were obtained from Roche Molecular Biochemicals. All cell-culture reagents were from Invitrogen (Stockholm, Sweden). All other reagents were from Sigma.

Cell culture

The cells were maintained at 37 °C under an atmosphere of 5 % CO₂ in DMEM (Dulbecco's modified Eagle's medium) with Glutamax II and 4.5 g/l D-glucose supplemented with penicillin/streptomycin and 5 % fetal-calf serum. ScN2a cells were generated as described previously [17], and were, together with non-infected N2a cells, generously provided by Professor Stanley B. Prusiner. N2a cells were also purchased from A.T.C.C. The ScN2a cells were routinely checked for their infection with scrapie, as described in [15].

Lectin chromatography and immunoprecipitation

Immunoprecipitation and purification of glycoprotein by lectin-coated beads were performed as described in [15]. Briefly, 2–4 mg of protein was incubated with 1 μ g of anti-IR β -subunit-specific antibody at 4 °C overnight, and for an additional 2 h with 20 μ l of Protein G-Sepharose beads. Glycoproteins were purified by incubating 2–4 mg of protein with 20 μ l of WGA beads for 3 h at 4 °C. Washed beads containing immunoprecipitated or lectin-purified proteins were solubilized in Laemmli sample buffer at 100 °C for 5 min. Proteins were separated by SDS/PAGE (7 % gels), immunoblotted with the indicated antibody and a secondary HRP-conjugated antibody, and visualized by ECL (enhanced chemiluminescence).

Detection of cell-surface IR

Cells (2×10^7) were incubated in DMEM without serum for 18 h, and then incubated in ice-cold buffer A [150 mM NaCl/1 mM MgCl₂/0.1 mM CaCl₂/20 mM Hepes/NaOH (pH 7.4)], containing 500 μ g/ml biotin-NHS-X (Calbiochem) for 40 min on ice. The cells were subsequently rinsed three times in cold buffer A supplemented with 50 mM NH₄Cl to quench the biotinylation reagent, and then lysed. The IR was immunoprecipitated as described above (using the anti-IR β antibody), subjected to SDS/PAGE and then electrotransferred to nitrocellulose membranes. The blots were probed with HRP-conjugated streptavidin and developed using ECL. The membranes were stripped in 0.1 M glycine, pH 2.5, for 7 min with constant shaking, and rehybridized further with the anti-IR β antibody. Densitometric measurements of surface-biotinylated IR levels were related to the total IR levels detected in the same lane. Densitometric analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health).

RT-PCR (reverse transcriptase-PCR) of IR-A and IR-B isoforms

Total RNA was isolated from N2a and ScN2a cells using TRI-reagent^{TM} (Sigma-Aldrich). cDNA was synthesized with a poly(dT)₁₈ primer and then amplified with mouse IR-specific primers: 5'-TGG-AGG-AGT-CTT-CAT-TCA-GG-3' (IR sense, located in exon 10) and 5'-AAT-GGT-AGA-GGA-GAC-GTT-GG-3' (IR anti-sense, located in exon 12) to generate PCR products of either 158 bp (IR-A) or 193 bp (IR-B). To control for any variability in sample preparation, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was also amplified with GAPDH-specific primers: 5'-GCC-CAG-AAC-ATC-ATC-CCT-GC-3' (GAPDH 647; sense) and 5'-GCC-TCT-CTT-GCT-CAG-TGT-CC-3' (GAPDH 1095; antisense) to yield a 449 bp fragment. Reactions were allowed to proceed for one cycle at 94 °C for

1 min, and then for 24, 28, 32 and 36 cycles consisting of 1 min at 94 °C, 2 min at 54 °C and 2 min at 72 °C, followed finally by a 5 min extension at 72 °C. Several numbers of amplification cycles were run to guarantee the amplification of IR and GAPDH fragments within the linear range. The products were analysed on 1.5 % (w/v) agarose gels and visualized by ethidium bromide staining.

Dephosphorylation of the IR β -subunit

Cells were starved for 18 h and treated with or without insulin (10 ng/ml in DMEM) for 5 min at 37 °C, washed once with PBS supplemented with 1 mM Na_3VO_4 , and thereafter lysed in extraction buffer [1 % (v/v) Triton X-100/150 mM NaCl/50 mM Hepes/NaOH (pH 7.6)/20 mM EDTA/10 mM sodium fluoride/30 mM sodium pyrophosphate/2 mM benzamide/1 mM Na_3VO_4] supplemented with protease inhibitors (1 mM PMSF, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ aprotinin and 1 $\mu\text{g}/\text{ml}$ pepstatin). Cell lysates from N2a (4 mg) and ScN2a (2 mg) cells were lectin-purified, and the WGA beads were washed 3 times with dephosphorylation buffer. WGA-purified IR was incubated with or without 5 units/ml CIAP in 50 mM Tris/HCl, pH 9.0/0.1 % Triton X-100 for 90 min at 37 °C, or 5 units/ml PAP (potato acid phosphatase) in 50 mM sodium citrate, pH 5.5/0.1 % Triton X-100 for 90 min at 37 °C. Dephosphorylated proteins were separated by SDS/PAGE (7 % gels) and hybridized with anti-IR β antibody, dehybridized and rehybridized with anti-phosphotyrosine antibody.

N-terminal sequence analysis of the IR β -subunit

Immunoprecipitated IR ($\approx 10 \mu\text{g}$), corresponding to 300 mg of cellular proteins from ScN2a cells, was electroblotted on to PVDF membranes and detected via Coomassie Blue staining. The protein band was cut and applied to a Procise cLC or HT sequencer (Applied Biosystems) for Edman degradation.

Enzymic deglycosylation of the IR α - and β -subunits

Enzymic deglycosylation was performed according to the recommendations in the pack-insert of PNGase F [cat. no. 1365193; Roche (www.roche-applied-science.com/pack-insert/1365185a.pdf)]. Immunoprecipitated IR, still adsorbed on to Protein G-Sepharose, from N2a (4 mg) and ScN2a (2 mg) cells was washed 3 times in wash buffer (same composition as extraction buffer, except that 0.1 % Triton X-100 was present), and then treated with 100 μl of 125 m-units/ml endo H or 125 units/ml PNGase F in 100 mM phosphate buffer, pH 5.5 or pH 7.0 respectively, containing 0.1 % Triton X-100, for 16 h at 4 °C (see Figure 4A). Deglycosylated IR was then washed 3 times in wash buffer, eluted in Laemmli sample buffer at 100 °C for 5 min, and subjected to Western blot analysis.

Alternatively, a large batch of denatured, immunoprecipitated IR was prepared in order to perform enzymic deglycosylation of denatured IR. Cellular protein (200 or 100 mg from N2a and ScN2a cells respectively) was incubated with 40 μg of anti-IR β antibody at 4 °C for 16 h, and then incubated for an additional 2 h with 100 μl of Protein G-Sepharose beads. The beads were washed 3 times in wash buffer, and IR was eluted in 200 μl of 0.5 % (w/v) SDS and 25 mM dithiothreitol for 5 min at 100 °C. Denatured IR (2.5 μl) was diluted 40-fold in 50 mM phosphate buffer, pH 7.2, containing 0.1 % Triton X-100, and then incubated with 10 units/ml PNGase F, 20 m-units/ml neuraminidase or 5 m-units/ml O-glycanase, or a combination of these, for 16 h at 24 °C (see Figures 4B and 4C). The reaction was stopped by the addition of 35 μl of 4 $\times$ Laemmli sample buffer at 100 °C for

Table 1 Table summarizing previous findings

WB, Western blot; B_{max} was calculated using the equation $B_{\text{max}} = B(K_d + [L^*])/[L^*]$, where $[L^*]$ (the concentration of the ligand) was 40 pM, K_d both for N2a and ScN2a cells was 1 nM, and B_{max}/IR represents the calculated number of binding sites/amount of immunoreactive IR in control wells.

Cell type	IR (WB) (%)	K_d (nM)	B_{max} (fmol/mg)	B_{max}/IR (%)
N2a	100 $\pm$ 6	1 $\pm$ 0.12	72 $\pm$ 9	100 $\pm$ 9
ScN2a	200 $\pm$ 1	1 $\pm$ 0.09	50 $\pm$ 6	35 $\pm$ 5

5 min. Deglycosylated IR (40 μl , corresponding to 0.5–1 mg of protein) was subjected to Western blot analysis.

Chemical deglycosylation with TFMSA (trifluoromethane sulphonic acid)

Acid deglycosylation with TFMSA was performed according to instructions provided in the GKK-500, Glyko® GlycoFree™ Chemical Deglycosylation Kit Manual (<http://www.prozyme.com/glyko/detection.html>). A sample (20 μl) of the denatured, immunoprecipitated IR (from the large batch described in the Enzymic deglycosylation of the IR α - and β -subunits section above), corresponding to ≈ 20 mg of cellular protein and 100 μg of insulin (added as a carrier), was precipitated with 1.3 ml of 90 % cold acetone at -70 °C, and collected by centrifugation at 17 000 g (15 min at 0 °C). The pelleted IR was transferred to glass vials, thoroughly freeze-dried, placed in a solid- CO_2 /ethanol bath and capped with a Teflon-lined septum and cap. TFMSA/toluene (50 μl ; 8:1, v/v) was added through the septum, and incubated at -20 °C for 4 h. Following neutralization with 150 μl of pyridine/ H_2O /methanol (3:1:1, by vol.), the addition of 400 μl of 0.5 % NH_4HCO_3 resulted in the formation of a precipitate that contained most of the protein. The precipitate and aqueous portion were collected by acetone precipitation, as described above. The pelleted deglycosylated IR was solubilized in 4 $\times$ Laemmli sample buffer, boiled and vortex-mixed for 10 min, before Western blot analysis.

RESULTS

Localization of IR in ScN2a cells

As summarized in Table 1, we have previously shown that scrapie infection induces a 2-fold increase in the level of IR protein in ScN2a cells, as measured by Western blotting, without a corresponding increase in IR mRNA. A similar increase in the IR protein level was also found in a neuroblastoma cell line (N1E-115) that was independently infected with scrapie (ScN1E-115) [15]. Displacement binding studies with ^{125}I -insulin showed that IRs in N2a and ScN2a cells exhibit the same affinity, with K_d values of 1 nM. However, the number of specific ^{125}I -insulin binding sites (B_{max}), as extrapolated from the binding of 40 pM ^{125}I -insulin to attached cells, showed a 30 % decrease in ScN2a compared with N2a cells. Correlating the number of binding sites in each well to the elevated IR protein levels detected by Western blotting in control wells (B_{max}/IR) revealed a 65 % decrease in binding sites in ScN2a compared with N2a cells. In addition to this contradiction, the IR β -subunit in ScN2a cells migrated with an apparently reduced molecular mass of ≈ 85 kDa, as compared with 95 kDa in N2a cells [15]. Together, these findings indicate a modified post-translational processing of the IR in ScN2a cells, possibly so severe that a fraction of the IR in ScN2a cells no longer

Figure 1 Determination of surface-localized IR

The amount of surface IR was determined by biotinylation of cell surface proteins, followed by anti-IR β immunoprecipitation (IP) of N2a (4 mg of protein) and ScN2a (2 mg of protein) cell lysates. Anti-IR β immunoprecipitates were separated by SDS/PAGE (7% gels) and immunoblotted with anti-IR β antibody (left lanes). After stripping, the blot was rehybridized with HRP-conjugated streptavidin (SA-HRP) and visualized by ECL (right lanes). Shown is one representative experiment out of three.

recognizes and binds ^{125} I-insulin, as illustrated by the 65% loss in binding sites despite unchanged receptor affinity.

In the previous study [15], the IR was detected in the WGA-purified fraction or whole-cell lysate by Western blotting, a method which does not distinguish between intracellular receptors and receptors integrated in the plasma membrane. To examine whether the increased IR protein level in ScN2a cells could be detected on the cell surface, i.e. whether the IR is correctly processed and transported to the cell surface or whether the increased IR levels in ScN2a cells reflect an increased intracellular accumulation, surface biotinylation was performed. N2a and ScN2a cells were labelled with biotin-X-NHS (a cell-impermeant protein reagent that biotinylates exposed lysine residues), followed by IR β immunoprecipitation and Western blotting. Immunoprecipitated IR from surface-biotinylated N2a (4 mg) and ScN2a (2 mg) cells was first immunoblotted with anti-IR β antibody. The membrane was stripped, and biotinylated IR was subsequently detected with HRP-streptavidin (Figure 1). There was no significant difference in the ratio of surface compared with total amount of immunoreactive IR between N2a and ScN2a cells ($99.9 \pm 2.7\%$, $n = 3$), when comparing the relative intensities of total IR β (left lanes) with that of surface-biotinylated IR β (right lanes) between the cell types (i.e. the ratio of the ratios of total IR/surface IR in ScN2a cells divided by total IR/surface IR in N2a cells was determined), although the number of surface-localized IRs in ScN2a cells was 2-fold

Figure 2 Presence of IR-IGF-1R hybrid receptors

Anti-IR β immunoprecipitates (IP) from N2a (4 mg of protein) and ScN2a (2 mg of protein) cells were separated by SDS/PAGE (7% gels) and immunoblotted with anti-IR β antibody. The blot was dehybridized and rehybridized with anti-IGF-1R β -chain antibody. This experiment shown is representative of two independent experiments.

higher than that found in N2a cells (half the amount of ScN2a protein was used for immunoprecipitation). The proportionately increased level of surface IR in ScN2a cells was an unexpected result, since binding studies clearly showed an important decrease in binding sites. Also note that the IR α -subunits in ScN2a cells migrate significantly faster, with an apparent molecular mass of 128 kDa compared with 136 kDa in N2a cells.

Presence of IR-IGF-1R hybrid receptors

In addition to IR, both N2a and ScN2a cells also express IGF-1R [16], and numerous studies have shown that in both human and rodent tissues and cell lines, expressing both types of receptors, hybrid receptors exist that are made up of one $\alpha\beta$ -heterodimer from an IR and one $\alpha\beta$ -heterodimer from the IGF-1R [18,19]. The functional importance of these receptors is not known, but numerous studies have shown that the hybrid receptor resembles the IGF-1R more than the IR, since it loses its high affinity for insulin, but retains high affinity for IGF-1 [19,20]. As the proportion of hybrid receptors appears to depend largely on the molar ratio of the IR and IGF-1R content [21], together with that fact that ScN2a cells express 4-fold-increased levels of IGF-1R [16] (in addition to the 2-fold increase in IR content), the level and ratio of IR-IGF-1R hybrids were determined. IR immunoprecipitates from N2a (4 mg of total protein) and ScN2a (2 mg of protein) cells were separated by SDS/PAGE, and hybridized with the IR β -specific antibody. Subsequently, the blot was stripped and rehybridized with the IGF-1R β antibody. As shown in Figure 2, the immunoprecipitated IR level in ScN2a cells was twice that in N2a cells (half the amount of ScN2a protein was used for immunoprecipitation), and rehybridizing the blot with the

Figure 3 Dephosphorylation of IR β -subunit

(A) Anti-IR β immunoprecipitates from N2a and ScN2a cells were dephosphorylated with PAP or CIAP, as described in the Materials and methods section, and separated further by SDS/PAGE (7% gels) and immunoblotted with anti-IR β antibody. (B) N2a (lanes 1 and 2) and ScN2a cells (lanes 3 and 4) (1×10^7 of each) were treated with 10 ng/ml insulin for 5 min at 37 °C and subjected to anti-IR β immunoprecipitation. Anti-IR β immunoprecipitates were treated with (lanes 2 and 4) or without (lanes 1 and 3) PAP, and separated by SDS/PAGE (7% gels) and immunoblotted with anti-phosphotyrosine [anti-P(Y)] antibody. The experiment shown is representative of three independent experiments.

anti-IGF-1R β antibody showed a parallel increase in IGF-1R immunoreactivity in the IR immunoprecipitate. This clearly shows that, although the ratio of IR-IGF-1R hybrids in N2a and ScN2a cells remains unchanged, the total amount of hybrid receptors in ScN2a cells is twice that in the uninfected cells. This finding could explain the decrease in ^{125} I-insulin-binding sites in ScN2a cells despite the unchanged surface biotinylation, i.e. plasma-membrane localization of IR in these cells.

Changed electrophoretic mobility of IR subunits in ScN2a cells

In addition to the increased level of IR protein in ScN2a and ScN1E-115 cells, the IR α - and β -chains displayed an increased electrophoretic mobility in SDS/PAGE, corresponding to molecular masses of 85 and 128 kDa, compared with 95 and 136 kDa respectively in N2a cells (see Figure 1 and [15]). In our previous study [15], the shift in molecular mass of the IR α -subunit was not as significant, due to a different gel composition and shorter electrophoresis. This change in migration may be due to alteration(s) in various post-translational modifications, including phosphorylation, proteolytic processing/degradation and glycosylation, each of which is discussed below.

Phosphorylation

To examine whether the apparent decreased molecular mass of the IR β in ScN2a cells is due to changes in phosphorylation, since the electrophoretic mobility of a protein may differ significantly, depending on its degree of tyrosine- and serine/threonine-phosphorylation [22], lectin-purified IR from N2a and ScN2a cells was dephosphorylated by CIAP or PAP, followed by Western blotting with the anti-IR β antibody (Figure 3A). To verify the enzymic dephosphorylation, lectin-purified IR from insulin-stimulated N2a and ScN2a cells was treated with or without PAP, and thereafter immunoblotted with an anti-phosphotyrosine antibody, demonstrating a complete dephosphorylation (Figure 3B). CIAP was equally efficient in dephosphorylating the phosphotyrosine residues (results not shown). However, the IR β -subunit from N2a and ScN2a cells still displayed the same

difference in electrophoretic mobility after treatment with either alkaline or acid phosphatase, indicating that the decreased molecular mass of IR β is not due to differences in tyrosine phosphorylation. As numerous studies have shown that both PAP and CIAP dephosphorylate phospho(serine/threonine) [23,24] as well as phosphotyrosine residues, this conclusion probably also holds for serine/threonine phosphorylation, although not verified in the present study.

Proteolytic processing/degradation

As the shift in electrophoretic mobility of IR β in N2a and ScN2a cells was still maintained after dephosphorylation, a possible explanation could be that the difference is due to changed proteolytic processing. Interestingly, a decrease in the molecular mass of the IR β -chain due to a C-terminal proteolytic degradation has been observed in several midbrain regions of Parkinsonism patients [25]. However, a plausible C-terminal processing of IR β in N2a and ScN2a cells is excluded, since the anti-IR β antibody (sc-711 from Santa Cruz Biotechnology), which was used for immunoprecipitation and immunoblotting, is directed against a peptide contained in the last 20 amino acids in the C-terminus of mouse IR β . To elucidate whether the N-terminus of IR β is intact in ScN2a cells, sequence analysis of immunoprecipitated IR β was performed. The first 10 cycles of sequence analysis resulted in LEEVGNVTA, which is identical to the expected N-terminal sequence SLEEVGNVTA of IR β , contained in positions 753–1372 of the IR proreceptor. The only mismatch in the experimentally obtained sequence was the absence of the first serine residue. However, as the following residues were identical, the absence of only one amino acid residue cannot explain the ≈ 10 kDa shift in the apparent molecular mass of IR β in N2a and ScN2a cells. It was therefore concluded that proteolytic processing of IR β in ScN2a cells does not provide the explanation for the altered electrophoretic migration.

Glycosylation

During its biosynthesis, the IR α - and β -subunits undergo intricate N-linked glycosylation. In addition, the β -subunit has also been shown to contain an O-linked carbohydrate. In order to determine whether the difference in molecular mass between the N2a and ScN2a IR β was due to differences in N-linked glycosylation, the IR was immunoprecipitated and enzymically deglycosylated with PNGase F and endo H. PNGase F cleaves the innermost N-acetylglucosamine of most N-linked glycan groups, whereas endo H cleaves the chitobiose core of N-linked high-mannose and some N-linked hybrid oligosaccharides, leaving the innermost N-acetylglucosamine intact [26]. Treatment with PNGase F and endo H significantly increased the electrophoretic mobility of the IR β from both N2a and ScN2a cells, although endo H was less efficient, indicating a smaller amount of endo H-releasable high-mannose or hybrid-type oligosaccharides in the mature β -subunit. However the difference between IR β from N2a and ScN2a cells still remained (Figure 4A), indicating that the difference is not due to changes in high-mannose or complex-type N-linked glycans. In a more detailed analysis, immunoprecipitated IRs from N2a and ScN2a cells were treated with neuraminidase alone (Figure 4B), or in combination with PNGase F and O-glycosidase (Figure 4C) in order to remove the terminal sialic acid residues, which may inhibit the accessibility of PNGase F and inhibit the enzymic activity of O-glycanase [27]. Following neuraminidase treatment, both IR α - and β -chains migrated faster, although the shift in apparent molecular mass still remained, indicating a similar capping with sialic acid of IR from N2a and ScN2a

Figure 4 Enzymic and chemical deglycosylation of IR

(A) Anti-IR β immunoprecipitates from N2a and ScN2a cells were treated with PNGase F (PNG-F) and endo H, as described in the Materials and methods section, separated further by SDS/PAGE (7% gels) and immunoblotted with anti-IR β . Shown is one representative experiment out of three. (B) Denatured anti-IR β immunoprecipitates from N2a and ScN2a cells were treated with neuraminidase (Neur) alone or (C) in presence of PNGase F (PNG-F) and O-glycanase (O-Gly), or treated with anhydrous TFMSA alone for 4 h as indicated and described in the Materials and methods section. Samples were separated further by SDS/PAGE (7% gels) and immunoblotted with a mixture of anti-IR α and -IR β antibodies (B), anti-IR α (upper panel in C) and anti-IR β antibodies (lower panel in C). The experiment shown is representative of two independent experiments. N, N2a; S, ScN2a; IgG, heavy chain of anti-IR β antibody; the asterisk shows the non-specific band.

cells. Only after a simultaneous treatment with neuraminidase, O-glycanase and PNGase F was the reason for the different molecular masses of IR α - and β -chains in ScN2a cells conclusively revealed, caused by differences in N- and/or O-linked glycosylation (Figure 4C). This was verified further by chemical deglycosylation. Among all methods that have been developed for chemical deglycosylation, anhydrous TFMSA has been found to be the most successful reagent for cleaving N- and O-linked glycans non-selectively from glycoproteins, leaving the primary structure of the protein intact [28,29]. As shown in Figure 4(C), following simultaneous treatment with neuraminidase, O-glycanase and PNGase F (lanes 3 and 4) or acid treatment with anhydrous TFMSA (lanes 5 and 6), the aglyco-IR α (upper panel) and -IR β (lower panel) from N2a and ScN2a cells displayed identical electrophoretic migrations. The combined enzymic deglycosylation resulted in IR β -subunits with apparently lower molecular mass than the chemical deglycosylation. However, this difference in migration was probably due to remaining chemical impurities in the TFMSA-treated samples, since the IgG in those samples also migrated differently, with a slower electrophoretic rate.

DISCUSSION

We have previously demonstrated that ScN2a cells express 2-fold increased levels of IR, as measured by Western blotting, compared with uninfected cells. Despite this significant increase in IR protein, the specific ^{125} I-insulin-binding sites were decreased by 30% compared with N2a cells, when ^{125} I-insulin binding (40 pM) was performed on attached cells. In addition, the IR α - and β -subunits from ScN2a cells migrate with apparent decreased molecular masses of 8–10 kDa respectively, compared with those from N2a cells.

The first question to resolve was whether the trafficking and/or localization of the IR was aberrant in ScN2a cells, or alternatively, whether the increased IR levels in ScN2a cells could be the result of inhibited degradation of internalized/recycled receptors. Surface biotinylation with the impermeant reagent biotin-X-NHS, followed by anti-IR β immunoprecipitation, revealed no difference in the ratio of surface-localized IR as compared with total amount of IR receptors between N2a and ScN2a cells (Figure 1), indicating that the 'non ^{125} I-insulin binding' pool of IR in ScN2a cells was not localized intracellularly, but expressed at the plasma membrane.

In addition to the typical IR, the majority of cells expressing significant levels of IR and IGF-1R also produce IR-IGF-1R hybrid receptors [21,30,31]. These hybrid receptors are formed in the endoplasmic reticulum, where the IR and IGF-1R precursors encounter and become disulphide-bonded to one another, rather than to a homologous precursor. It is suggested that IR-IGF-1R hybrid receptors are formed by random assembly, in which hybrid and typical receptors are formed with equal efficiency and in proportions determined by the molar ratio of each receptor type [18,21]; however, the fraction of hybrid receptors does not always correlate with the relative levels of IR and IGF-1R. In certain tissues, hybrid receptors form the most represented receptor subtype, and in the brain 50% of total IGF-1R was present as hybrid receptors [18]. Hybrid receptors have also been suggested to exist in sympathetic and sensory ganglion cells [32]. Although the biological role of these receptors is not yet known, functional studies have indicated that IR-IGF-1R hybrid receptors behave more like the IGF-1R than the IR, because they bind to and are activated by IGF-1 with much higher affinity than insulin [18,19].

To add one more dimension of complexity to these hybrid receptors, the IR exists as two isoforms, and depending on the IR isoform involved, the hybrid receptors will have different pharmacological and biological features. The two IR isoforms are the result of alternative splicing of exon 11 in a tissue-specific manner [33]. The IR-A (Ex11-) lacks, whereas the IR-B (Ex11+) contains, the sequence coding for 12 amino acids in the C-terminal part of the α -subunit [9]. The brain and also peripheral neurons show predominantly the IR-A type, which displays higher affinity for insulin compared with the peripheral IR-B type [8]. Thus a hybrid receptor containing IR-A binds insulin with 10–20-fold lower affinity than the IR, whereas a hybrid receptor containing IR-B hardly recognizes insulin at all, with an $IC_{50} > 100$ nM [20]. Because N2a cells express both IRs and IGF-1Rs, and ScN2a cells express 2-fold and 4-fold increased levels of IR and IGF-1Rs respectively, a possible scenario is that, in ScN2a cells, a larger number of hybrid receptors are formed than in N2a cells, with high affinity for IGF-1, but low affinity for insulin. In order to determine which IR isoform is expressed in N2a and ScN2a cells, cDNA was amplified with IR-specific primers located in exon 10 and exon 12, thus yielding PCR products of different size ($\pm$ Ex11). Both N2a and ScN2a cells expressed mainly the IR-A (results not shown), and therefore the resulting hybrid receptors are expected to exhibit a 10–20-fold lower affinity than homotypic IR.

We can show that, in N2a cells, a large proportion of IR is engaged as hybrid receptors, and in ScN2a cells twice the number of hybrid receptors are formed compared with N2a cells, even though the ratio of hybrid receptors measured against IRs is similar in N2a and ScN2a cells (Figure 2). This 2-fold increase in hybrid receptor content in ScN2a cells could be a possible explanation for the loss of ^{125} I-binding sites in ScN2a cells. As the tracer concentration of ^{125} I-insulin used to detect binding sites was 40 pM, mainly homotypic IRs with a K_d of 1 nM will bind ^{125} I-insulin. Since a larger amount of the immunoreactive IRs in ScN2a cells is involved in hybrid receptors with a 10–20-fold lower affinity, the result will be an apparent loss of ^{125} I-binding sites, although the receptors are still detectable by Western blotting. We are currently investigating the ratios of IR/hybrid receptor and IGF-1R/hybrid receptor in N2a and ScN2a cells, to confirm this hypothesis. The functional significance of the increased numbers of IR and hybrid receptors remains to be established; however, as hybrid receptors function as IGF-1Rs, the increased hybrid receptor formation would amplify the IGF-1 responsiveness in ScN2a cells.

In our attempts to ascertain why IR β in ScN2a cells migrates faster on SDS/PAGE than in N2a cells, we have taken into account several possibilities, including changes in the phosphorylation, proteolytic processing or glycosylation of IR β in ScN2a cells. Our results show that this change is neither due to differences in phosphorylation nor to proteolytic processing of IR β in ScN2a cells, but due to differences in the glycosylation of IR β . The decreased molecular mass of the IR α -subunit in ScN2a cells is also due to aberrant glycosylation, since combined enzymic and chemical deglycosylation produced aglyco-IR α from ScN2a cells migrating identically with the aglyco-IR α from N2a cells (Figure 4C).

Elucidation of the primary structure of the IR has identified 14 and 4 potential N-linked glycosylation sites on the α - and β -subunit respectively [9]. The importance of N-glycosylation of the IR proreceptor for correct IR processing and trafficking was demonstrated at an early stage using tunicamycin, an antibiotic which blocks N-linked glycosylation, resulting in intracellular accumulation of an aglyco-proreceptor [10]. Later, by studies using site-directed mutagenesis of the individual N-linked glycosylation sites, it was shown that mutation of the first or

second pair of N-glycosylation sites in the α -subunit leads to accumulation of the proreceptor in the endoplasmic reticulum [34]. Individual mutations have no effect on processing or binding affinity [34,35].

In the β -subunit, mutation of the asparagine residue within the four potential N-glycosylation sites has no effect on processing [36,37]. However, mutations of the third and fourth N-glycosylation sites in the β -subunit inhibited insulin-induced tyrosine kinase activity, although the affinity was unaltered. These results indicate that both the number and location of specific N-glycosylation sites in the IR differentially affect processing and gain of function.

Our results show that, although the IR α - and β -chains are aberrantly glycosylated in ScN2a cells, the insulin proreceptor is correctly glycosylated for further processing and trafficking to the cell surface, as shown by the similar ratio of surface localized compared with total immunoreactive IR in N2a and ScN2a cells (Figure 1). This contradicts previous findings indicating that the crucial N-glycosylation sites are located in the α -subunit of the IR proreceptor [34–36]. However, in a more recent study it was shown that there are many redundancies in the IR α glycosylation sites, and that every site can be mutated individually without loss of receptor processing, cell-surface expression or ligand binding, although certain combinations inhibited correct folding and processing [38].

An alternative explanation for the loss of ^{125} I-insulin binding sites in ScN2a cells could be a significant decrease ($K_d > 20$ nM) or loss of binding affinity of the aberrantly glycosylated IR α -subunit, since the α -subunit contains the ligand-binding domain. However, this is not likely, because the insulin proreceptor acquires insulin binding function before it is proteolytically cleaved into α - and β -subunits of the mature IR and processed further and transported to the plasma membrane. Also, once the proreceptor has acquired ligand binding affinity, removal of its N-linked oligosaccharide chains with endo H has no effect on the ability of the proreceptor to bind insulin [39].

Brain or neuronal IRs are biochemically and immunologically distinct from peripheral IR, and one possible explanation to account for the presence of smaller IR α - and β -subunits in ScN2a cells is an induction of a neuronal 'glycosylation phenotype' by the scrapie infection. Brain IR contains α - and β -subunits of ≈ 120 kDa and 83–90 kDa respectively, as compared with peripheral IR, with α - and β -subunits of ≈ 135 kDa and 95 kDa respectively [12]. This discrepancy in molecular mass was attributed to differences in oligosaccharide content, specifically in the degree of complex-type glycans and terminal sialic acid, because the neuronal type IR was unaffected by treatment with neuraminidase, whereas the peripheral-type IR was neuraminidase-sensitive [12].

As far as the IR in the neuroblastoma N2a cells is concerned, it behaves like a peripheral-type IR, since the IR α - and β -subunits exhibit molecular masses of ≈ 135 kDa and 95 kDa respectively. In addition they were both neuraminidase-sensitive (Figure 4B), consistent with previous findings indicating a peripheral-type IR, at least from a glycosylation point of view, in various neuroblastoma cell lines [40]. Arguing against the hypothesis that the IR in ScN2a cells has gained a neuronal phenotype is the fact that both IR subunits in ScN2a cells are, as in N2a cells, still neuraminidase-sensitive.

Dolichol and dolichyl phosphate are key intermediates and putatively rate-limiting factors in the assembly and attachment of N-linked oligosaccharides [41,42]. Interestingly, changes in these intermediates have been reported in both scrapie-infected mice and Creutzfeldt–Jakob disease patients [43,44]. In addition, comparison of the composition of N-linked glycans of PrP^C and

PrP^{Sc} from scrapie-infected hamster revealed decreased levels of glycans with bisecting *N*-acetylglucosamine residues and increased levels of tri- and tetra-antennary sugars attached to PrP^{Sc} [45]. This is consistent with a decrease in the activity of *N*-acetylglucosaminyltransferase III, one of the enzymes controlling branching during maturation of the N-linked oligosaccharide. Together, these changes argue that prion formation may perturb the glycosylation machinery in prion-infected cells, resulting in other glycoproteins being glycosylated aberrantly. This is supported further by our finding that the IR in ScN2a cells is differently glycosylated. Further studies are needed to determine why only selected glycoproteins, e.g. the IR but not the IGF-1R β (Figure 2), are affected, as well as the significance of modified protein glycosylation in prion-infected cells.

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