

Inhibition of α -Glucosidases I and II Increases the Cell Surface Expression of Functional Class A Macrophage Scavenger Receptor (SR-A) by Extending Its Half-life*

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The class A scavenger receptor (SR-A) is a multifunctional trimeric membrane glycoprotein involved in atherogenesis. The mature receptor can mediate the binding and internalization of a number of specific ligands, including modified low-density lipoprotein. We have investigated the effects of inhibiting *N*-glycan processing on SR-A expression, distribution, and activity in the murine macrophage cell line RAW264.7. We have found that SR-A normally interacts with calnexin in the endoplasmic reticulum and in its mature form carries complex *N*-glycans. The imino sugar, *N*-butyldeoxynojirimycin (NB-DNJ) is an inhibitor of the *N*-glycan processing enzymes α -glucosidases I and II. Following NB-DNJ treatment SR-A became Endo H-sensitive, consistent with inhibition of *N*-glycan processing. A dose-dependent increase in cell surface expression of SR-A was observed in response to NB-DNJ treatment. The receptor on inhibitor-treated cells was still functional because the increased surface expression resulted in a proportional enhancement in the endocytosis of the ligand, acetylated low-density lipoprotein. The expression of SR-A on NB-DNJ cultured cells was further enhanced by co-treatment with interferon- γ . Quantitative reverse transcriptase-PCR analysis did not show a significant difference in the amount of SR-A mRNA in NB-DNJ-treated RAW264.7 cells. However, the half-life of SR-A protein was significantly increased. These data indicate the retention of glucosylated *N*-glycans does not result in gross misfolding and degradation of this receptor or prevent its transport to the cell surface. SR-A interacts with calnexin and when the association is prevented changes in the recycling kinetics and rate of turnover of the receptor result, leading to enhanced cell surface expression.

The macrophage class A scavenger receptor (SR-A)¹ is a trimeric integral membrane glycoprotein that displays unusu-

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¹ The abbreviations used are: SR-A, class A scavenger receptor; NB-DNJ, *N*-butyldeoxynojirimycin; IFN- γ , γ -interferon; acLDL,

ally broad ligand binding properties (1). SR-A can recognize a number of ligands, including chemically modified molecules (2–4). It is the uptake of one of these ligands, modified low-density lipoprotein (LDL), that promotes the conversion of monocyte-derived macrophages (M ϕ) into foam cells that characterize atherosclerosis (5). Unlike endocytosis via the LDL receptor, intracellular cholesterol levels do not down-regulate SR-A-mediated uptake of modified LDL, and cholesterol ester accumulation leads to the development of foam cells (3). This process is considered to be critical for disease progression and the important contribution of SR-A has been delineated through studies of atherogenesis in SR-A-deficient mice (6).

There are two major isoforms of SR-A, which are derived by alternative splicing (3, 7). Each contains six predicted structural domains: cytoplasmic, transmembrane, spacer, α -helical coiled coil, collagenous, and an isoform-specific carboxyl terminus. They differ only at the COOH terminus (3). Type II SR-A is identical to the type I receptor, except that the 110-amino acid SRCR domain of SR-AI is replaced by a 6-amino acid COOH terminus. Type II scavenger receptor mediates the endocytosis of modified LDL and binding of other ligands with essentially the same affinity and broad specificity as the type I receptor (8, 9). Experimental investigations of the properties of a series of truncated and site-specific mutant receptors have identified structural domains and specific residues that are important for conferring biological activities (10, 11). For example, lysines within the carboxyl terminus of the collagenous domain of bovine SR-A are critical for the binding of modified lipoprotein (12) and a histidine residue within the α -helical coiled coil is required for pH-dependent ligand dissociation (13).

Despite these extensive structure-activity studies of the molecule, there have been no reports of the potential significance of secondary modifications on the biochemical and biological properties of SR-A. The predicted protein sequence of human SR-A contain seven *N*-linked oligosaccharide consensus sequences, three of which lie within the extracellular spacer domain and four within the α -helical coiled coil (14) (Table I). A comparison across human, mouse, bovine, and rabbit SR-A sequences reveals that five sites (Asn¹⁰², Asn¹⁴³, Asn¹⁸⁴, Asn²⁴⁹, and Asn²⁶⁷) are absolutely conserved (9). To gain further insight into the biology of SR-A, we have characterized the effects of

acetylated low density lipoprotein; PNGase F, peptide-*N*-glycosidase F; Endo H, endo- β -*N*-acetylglucosaminidase H; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; RT, reverse transcription; LDL, low density lipoprotein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

the modulation of *N*-glycan processing on SR-A expression, distribution, and activity.

N-Linked glycosylation of nascent proteins involves the transfer of a precursor $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ from dolichol to the asparagine residue of the Asn-Xaa-Ser/Thr glycosylation sequon of the protein. The oligosaccharide is subsequently processed by the sequential action of trimming glycosidases in the lumen of the endoplasmic reticulum, prior to the elaboration of hybrid and complex type oligosaccharide structures in the Golgi apparatus. Trimming is initiated by endoplasmic reticulum α -glucosidase I, which releases the outer α -1,2-linked glucose residue from the oligosaccharide, followed by removal of the α -1,3-linked second and third glucose residues by α -glucosidase II. Several compounds that inhibit α -glucosidases I and II have been identified, including deoxynojirimycin (15) and its *N*-alkylated derivative *N*-butyldeoxynojirimycin (NB-DNJ) (15). In the presence of these imino sugars, immature glucosylated *N*-glycans are retained and therefore hybrid and complex type oligosaccharide synthesis is prevented (16). Glucosidase inhibitors have been successfully used as tools to probe the requirement of an interaction with the endoplasmic reticulum-resident lectin calnexin to achieve protein folding, a process that is primarily dependent upon a monoglucosylated *N*-glycan structure generated by the action of α -glucosidase II. For example, tyrosinase, a key enzyme in melanin biogenesis was catalytically inactive when synthesized in the presence of NB-DNJ, but was transported correctly to the melanosome (17). Its inactivity was because of misfolding that occurred in the absence of the normal interaction with calnexin, resulting in an inability to incorporate copper into its active site (17). It should be noted that recent evidence has revealed that in certain circumstances calnexin can display lectin-independent activities (18).

It is not possible to predict the precise effects of inhibiting *N*-glycan processing and must be determined on a case by case basis. We therefore examined the effects of culturing the murine macrophage cell line RAW264.7, which expresses significant levels of functional SR-A (19), in the presence of NB-DNJ. We found that SR-A usually carries complex *N*-linked carbohydrate structures, but addition of the imino sugar resulted in the predicted retention of glucosylated *N*-glycans that prevented association with calnexin. We observed correct trafficking of receptors to the cell surface, together with significantly enhanced levels of expression that led to increased endocytosis of acetylated (ac) LDL. We observed synergistic effects when cells were co-treated with NB-DNJ and the cytokine interferon- γ (IFN- γ). The increased expression was not because of regulation of transcription but was the result of increased receptor half-life. These data suggest that the interaction with calnexin may be a pre-requisite for correct folding of this protein that affects the turnover rate of this receptor.

EXPERIMENTAL PROCEDURES

Materials—NB-DNJ was purchased from Toronto Research Chemicals (Toronto, Canada). The rat anti-mouse scavenger receptor monoclonal antibody (2F8) was purchased from Serotech (Oxford, United Kingdom). Endo- β -*N*-acetylglucosaminidase H (Endo H), *N*-glycosidase F (PNGase F), and sialidase were obtained from Glyko (Upper Heyford, UK). Murine IFN- γ was obtained from R&D Systems (Abingdon, UK) and Tran³⁵S-labelTM, 1100 Ci/ml from ICN (Thame, UK). Gamma-BindTM Plus SepharoseTM was purchased from Amersham Biosciences. Sulfosuccinimidobiotin was purchased from Pierce. Horseradish peroxidase-conjugated extravidin was purchased from Sigma. Anti-calnexin monoclonal antibody was from Bioquote (York, UK).

Cell Culture—RAW-264.7 murine macrophage cells (from ATCC) were cultured in RPMI 1640 medium supplemented with 50 IU/ml of penicillin G, 50 $\mu\text{g/ml}$ streptomycin, 2 mM glutamine (all from Invitrogen), and 10% fetal calf serum (Sigma) and maintained at 37 °C with 5% CO₂. For radiolabeling experiments cells were placed in methionine-

and cysteine-free RPMI 1640 medium obtained from ICN (Thame, UK).

Treatment of RAW 264.7 Cells with Imino Sugar and Co-treatment with IFN- γ —NB-DNJ and IFN- γ were dissolved in double distilled, endotoxin-free water, filter-sterilized (0.2 μm), and added to the cells to the required final concentration. The cells were incubated in the presence of the imino sugar for the duration of the particular experiment. In brief, RAW 264.7 cells were cultured in the absence or presence of NB-DNJ for 2 weeks, and then co-treated with IFN- γ (100 units/ml) for 48 h at 37 °C.

Pulse-Chase Analysis—RAW 264.7 cells were cultured in the presence or absence of NB-DNJ (2 mM) at 37 °C for 2 weeks and subsequent co-incubation with IFN- γ for the following 48 h. Cells were harvested, washed three times with phosphate-buffered saline, 0.1 M (pH 7.2) (PBS), and resuspended in methionine- and cysteine-free RPMI 1640 medium supplemented with 5% dialyzed fetal calf serum and IFN- γ for 1 h. The cells were pulsed for 30 min with 300 $\mu\text{Ci/ml}$ Tran³⁵S-label. Cells were washed three times with PBS and incubated in "chase" medium consisting of 10% fetal calf serum supplemented with 1 mM unlabeled methionine, 4 mM unlabeled cysteine, and IFN- γ . NB-DNJ (2 mM) was maintained in the pulse and chase medium of treated cultures. After incubation for the indicated times, cells were washed five times with ice-cold PBS, then lysed on ice in 150 mM NaCl, 10 mM EDTA, 10 mM Na₃N, 10 mM Tris (pH 8.0), 1 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide, and 1% Nonidet P-40. Cell lysates were centrifuged at 13,000 $\times g$ for 20 min to remove debris. Supernatants were stored at -70 °C until used.

Immunoprecipitation and Endoglycosidase H Treatment—³⁵S-Labeled or unlabeled cell lysates were precleared with GammaBindTM Plus SepharoseTM for 2 h at room temperature. Antigen was precipitated overnight at 4 °C by incubation with 10 $\mu\text{g/ml}$ anti-mouse SR-A monoclonal antibody (2F8) and GammaBindTM Plus SepharoseTM, washed 6 times with 10 mM Tris (pH 8.0); 3 times in 500 mM NaCl, 0.5% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, 0.05% (w/v) SDS; twice with 150 mM NaCl, 0.5% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, 0.05% (w/v) SDS; once with 0.05% (w/v) SDS. The beads were resuspended in 50 mM Tris (pH 6.8) then boiled for 5 min in 5 \times Laemmli sample buffer with 5% (v/v) 2-mercaptoethanol. The supernatant was separated by 10% SDS-PAGE.

Samples for digestion with Endo H were eluted from the solid phase by heating at 100 °C for 5 min with 50 μl of 100 mM sodium citrate phosphate (pH 5.5), containing 0.2% SDS, 0.1 M 2-mercaptoethanol. The boiled sample was split into equal aliquots and incubated at 37 °C overnight in the presence or absence of 2 milliunits of Endo H. Digestion was terminated by the addition of SDS reducing sample buffer with heating at 100 °C for 5 min and separated by 10% SDS-PAGE. Gels were fixed and impregnated with AmplifyTM (Amersham) 15 min before drying and exposure to film at -70 °C.

Glycosidase Treatment of SR-A—The material that bound to protein G-Sepharose was treated with PNGase F and Endo H as described by the manufacturer. In brief, the beads were washed and boiled for 5 min in 10 μl of 0.2% (w/v) SDS and 1% (v/v) 2-mercaptoethanol. The volumes were adjusted to final detergent concentrations of 0.1% (w/v) SDS and 1% (v/v) Nonidet P-40 and incubated at 37 °C for 72 h in the presence of 15 units/ml PNGase F and at 37 °C overnight in the presence of 1 unit/ml Endo H or sialidase 100 milliunits/ml, respectively.

Immunoblotting—Cell lysates were subjected to 10% SDS-PAGE gel separation and electroblotted onto nitrocellulose membranes (ImmobilonTM-P transfer membrane, Millipore Corp.). Membrane was blocked for 1 h at room temperature in PBS containing 5% (w/v) nonfat powdered milk and 0.1% Tween 20 prior to the addition of the anti-mouse SR-A antibody. The membrane was washed and then incubated with peroxidase-conjugated goat anti-mouse IgG antibody (Sigma) diluted in blocking buffer for 1 h. Antibody binding was detected by enhanced chemiluminescence (ECL; Amersham Biosciences).

Flow Cytometry—Cells were harvested by centrifugation and resuspended in PBS, containing 0.02 M Na₃N, 0.1% bovine serum albumin (FACS buffer). Cells (1×10^6) were stained with fluorescein isothiocyanate-conjugated 2F8, washed, and resuspended in FACS buffer containing 2 $\mu\text{g/ml}$ propidium iodide (Sigma). Samples were analyzed using a FACScan flow cytometer (BD Biosciences). Dead cells were excluded from the analysis on the basis of differential uptake of propidium iodide. Data were collected on 10^4 viable cells and plotted on a four-decade log₁₀ scale of increasing fluorescence intensity on the x axis. To quantify copy number of surface receptors per cell, fluorescein-labeled quantitative microbead standard (BD Biosciences) were included within assays. To determine total receptor expression, cells were fixed with 4% paraformaldehyde (10 min on ice), washed three times with PBS, and incu-

bated with 0.5% saponin in PBS (10 min at room temperature) prior to incubation with antibody.

Quantitation of DiI-AcLDL Uptake—Cells were incubated in 10 μ g/ml DiI (1,1'-dioctadecyl-1-3, 3, 3',3'-tetramethylindocarbocyanine perchlorate)-labeled acLDL (DiI-acLDL) (Autogen Bioclear, Calne, UK) for 2 h at 37 °C in complete medium. Cells were washed three times in PBS and analyzed by flow cytometry.

Quantitative RT-PCR—All equipment and reagents were supplied by Applied Biosystems (Warrington). Primer and probe sequences that recognize both SRA-1 and SRA-2 transcripts were designed using Applied Biosystems Primer express software from the murine macrophage scavenger receptor sequence (9). The forward primer used was tggtc-cacctggtgctcc, the reverse primer was acctccaggggaagcaattt, and the probe was ataagaggtattccaggtgttaaggtgatcggg. Primer pairs were tested using conventional RT-PCR to confirm a single band of the expected amplicon size (not shown). cDNA was synthesized using 200 ng of total RNA (prepared using RNAeasy kit, Qiagen) in a 10- μ l reaction volume using RT Gold reagents according to the manufacturer's instructions. PCR was performed in a 25- μ l reaction using Taqman Universal Master mixture containing 100 nM each primer and 50 nM probe. For each reaction the equivalent of 20 ng of total RNA (1 μ l of cDNA) was used. Reactions were run on an Applied Biosystems PRISM 7700 Sequence Detection System, in duplicate, and quantified against a relative standard curve made from a serially diluted stock RNA containing the target sequence. Cycling conditions were 50 °C for 2 min, 95 °C for 10 min followed by a 40-cycle amplification phase of 95 °C for 15 s and 60 °C for 60 s. The amount of total RNA input into each RT reaction was assayed by measuring the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each cDNA sample using the Rodent GAPDH Taqman kit. Data were calculated as relative SRA mRNA expression, normalized to GAPDH.

SR-A Interaction with Calnexin—Lysates were prepared from RAW 264.7 cells cultured in the presence or absence of NB-DNJ (2 mM), then immunoprecipitated with anti-calnexin antibody, eluted, run on SDS-PAGE, and analyzed by Western blotting using 2F8 antibody.

Surface Biotinylation—RAW264.7 cells (1×10^6 /ml) were cultured in the presence or absence of 2 mM NB-DNJ with IFN- γ . The cells were washed twice in PBS containing 10% bovine serum albumin (PBS, 10% bovine serum albumin) and once in PBS. The cells were resuspended in 4.5 ml of PBS containing 1.5 mg of sulfo-succinimidobiotin (Pierce) and incubated at 37 °C for 30 min. The cells were washed as before and then returned to culture medium and incubated as indicated. At the indicated times, cells were incubated with 2F8 before lysis (cell-surface protein) or lysed immediately (total protein). The cell lysates were immunoprecipitated with 2F8 as above and analyzed by SDS-PAGE/Western blotting. Blots were developed with appropriate secondary antibodies and horseradish peroxidase-conjugated extravidin by ECL.

RESULTS

Effects of NB-DNJ on the Electrophoretic Mobility of SR-A—RAW264.7 cells were cultured in the presence or absence of different concentrations of NB-DNJ for 2 weeks prior to analysis by SDS-PAGE and Western blotting. A significant dose-dependent increase in the mobility of SR-A resulted from culturing the cells in NB-DNJ. Whereas the SR-A obtained from untreated cells migrated with a molecular mass of ~90 kDa, receptor from the RAW264.7 cells cultured with 2 mM NB-DNJ migrated as a species with an apparent mass of 66 kDa (Fig. 1A). The maximal increase in mobility was achieved at 2 mM NB-DNJ (Fig. 1A, lane 5) and was not enhanced by higher concentrations of drug (data not shown).

Glycosylation of SR-A—To define the glycosylation status of SR-A from untreated and NB-DNJ-treated cells, cell lysates were digested with Endo H or PNGase F. The de-glycosylated receptor (PNGase digested) had the predicted molecular mass of 45 kDa (9) (Fig. 1B). The receptor from untreated cells was fully Endo H resistant showing that the glycans carried by SR-A are all of the complex type (Fig. 1B). Endo H treatment of the lysate prepared from RAW264.7 treated with 2 mM NB-DNJ yielded a heterogeneous population of SR-A molecules that comprised at least 3 species (Fig. 1C). The lowest molecular mass species migrated as a 45-kDa protein, whereas the difference in molecular mass between the three species was ~2–3 kDa (Fig. 1C), consistent with receptors having zero, one,

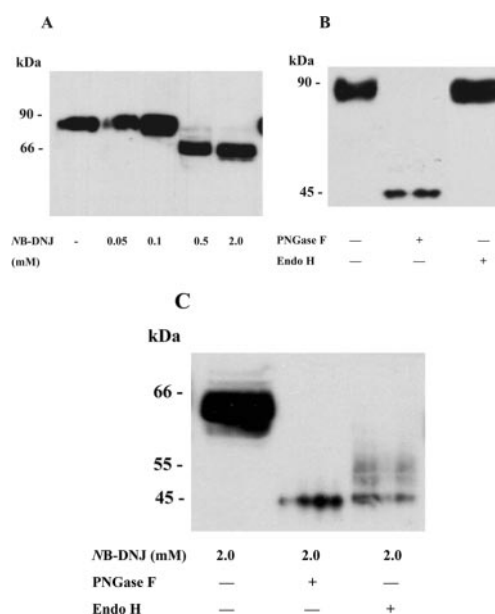


FIG. 1. A, SDS-PAGE mobility of SR-A prepared from RAW264.7 cells cultured in the presence of increasing concentrations of NB-DNJ. B, sensitivity of SR-A from untreated RAW264.7 cells to digestion with PNGase F and Endo H. C, sensitivity of NB-DNJ-treated SR-A to digestion with PNGase F and Endo H.

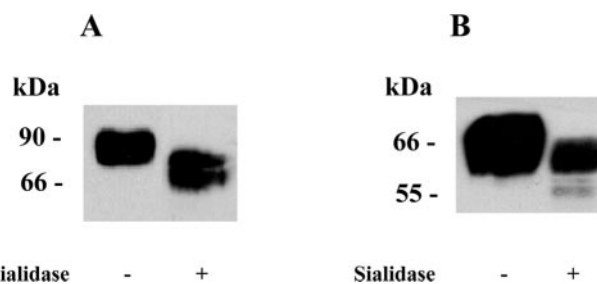


FIG. 2. Sensitivity of SR-A prepared from RAW264.7 cells cultured in the absence (A) and presence (B) of 2 mM NB-DNJ to digestion with sialidase.

TABLE I

Predicted sites of N-linked oligosaccharides on mammalian SR-A					
Asn residue	Protein domain	Mouse	Bovine	Human	Rabbit
Asn ⁸²	Spacer	-	+	+	+
Asn ¹⁰²	Spacer	+	+	+	+
Asn ¹⁴³	Spacer	+	+	+	+
Asn ¹⁸⁴	Coiled coil	+	+	+	+
Asn ²²¹	Coiled coil	-	+	+	+
Asn ²⁴⁹	Coiled coil	+	+	+	+
Asn ²⁶⁷	Coiled coil	+	+	+	+

or two N-glycosylation sites carrying oligosaccharides that could be because of incomplete digestion with Endo H or partial processing via Golgi endomannosidase (20). Sensitivity to sialidase digestion (Fig. 2A) indicated that SR-A normally carries complex N-glycans. The receptor from NB-DNJ-treated cells was, as predicted, predominantly resistant to sialidase digestion (Fig. 2B).

Pulse-Chase Analysis—The kinetics of SR-A N-glycan processing by untreated and NB-DNJ-treated RAW264.7 cells were investigated by pulse-chase analysis. At the 10-min time point (Fig. 3) SR-A was fully Endo H-sensitive in both untreated and drug-treated cells (Fig. 3, A and B). By 4 h, SR-A from untreated cells has been almost fully processed to an Endo H resistant form (Fig. 3A), whereas NB-DNJ-treated SR-A remained almost entirely fully Endo H sensitive, with only a

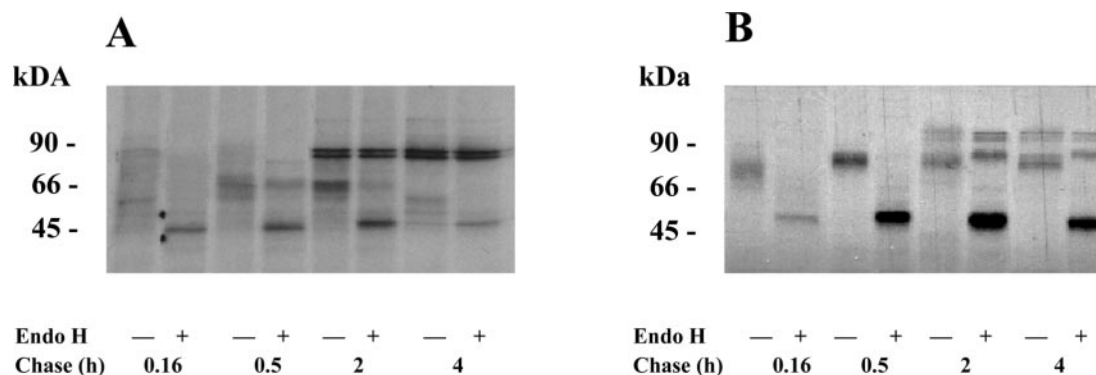


FIG. 3. Pulse-chase labeling of SR-A in RAW264.7 cells cultured in the absence (A) and presence (B) of 2 mM NB-DNJ. Chase time is stated in hours.

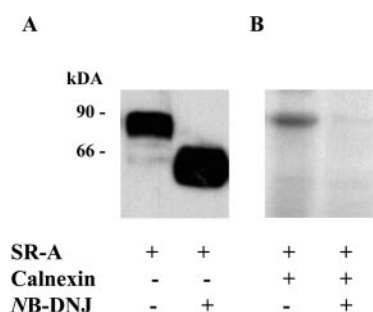


FIG. 4. Co-immunoprecipitation of SR-A and calnexin from RAW264.7 cells. Panel A, SR-A probed blot of SR-A immunoprecipitate from untreated and NB-DNJ (2 mM)-treated cells. Panel B, SR-A probed blot of calnexin immunoprecipitate from untreated and NB-DNJ (2 mM)-treated cells.

small fraction being Endo H resistant, presumably via flux of a small number of molecules through the Golgi endomannosidase pathway (20) (Fig. 3B).

SR-A Interacts with Calnexin—SR-A immunoprecipitated from untreated and NB-DNJ-treated cells had molecular masses of 90 and 66 kDa, respectively. When samples were first immunoprecipitated with an anti-calnexin antibody and subsequently probed with anti-SR-A antibody, the scavenger receptor could be detected only from untreated cells (Fig. 4).

Effects of NB-DNJ on Cell Surface and Total Expression of SR-A—To investigate whether NB-DNJ-treatment influenced SR-A expression, binding sites of the anti-SR-A antibody were quantified by flow cytometry. Both NB-DNJ treatment and IFN- γ treatment increased cell surface staining with 2F8 monoclonal antibody when compared with untreated RAW264.7 cells (in the region of an approximate 2-fold increase) (Fig. 5A). When cells were exposed to the two agents simultaneously, there was a more marked augmentation of binding sites (15.4 ± 3.3 S.E., -fold relative to untreated cells) (Fig. 5A). In comparison, total cellular expression of SR-A was augmented 4.8 ± 1.1 S.E., -fold relative to untreated cells (Fig. 5C).

DiI-AcLDL Uptake by NB-DNJ-treated Cells—SR-A is involved in the cellular uptake of acLDL by macrophages (1). To study the functional properties of glycosylated SR-A, endocytosis of the fluorescently labeled SR-A ligand DiI-acLDL by untreated, NB-DNJ-treated, and NB-DNJ plus IFN- γ -treated RAW264.7 cells was measured by flow cytometry. Cells treated with NB-DNJ alone showed a significant increase in DiI-acLDL uptake compared with untreated cells (Fig. 5C). The increase in endocytic activity of drug plus cytokine-treated cells (13.2 ± 4.2 S.E., -fold increase in comparison to untreated) approximated that of SR-A surface expression (15.4 ± 3.3 S.E. greater in NB-DNJ plus IFN- γ -treated RAW-264.7 cells (Fig. 5A).

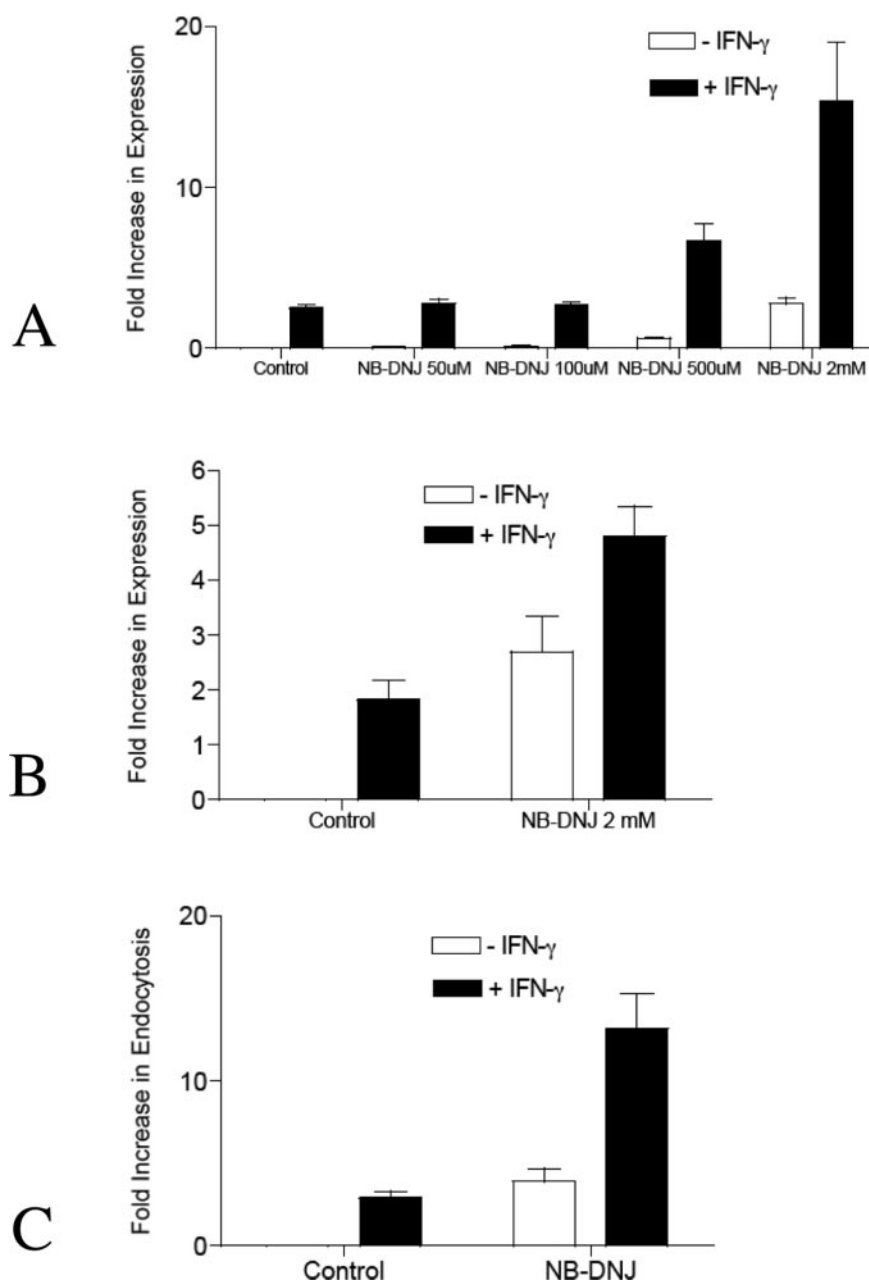
Quantitative RT-PCR Analyses of NB-DNJ and Untreated Cells—To investigate the mechanism of increased cell surface expression of SR-A, SR-A mRNA levels were determined by quantitative RT-PCR analyses in untreated and NB-DNJ-treated RAW264.7 cells. There was no significant difference in the relative amount of SR-A mRNA (as determined by C_t values and relative expression compared with that of GAPDH) between the untreated, drug-, and cytokine-treated cells (data not shown), suggesting that the mechanism of increased cell surface receptor expression did not arise at the level of receptor transcription.

Kinetics of Turnover of Cell Surface and Total Cellular SR-A—To determine whether the increase in expression of SR-A that accompanied exposure to the imino sugar reflected a change in the turnover rate of the receptor, we undertook pulse-chase analysis. These studies revealed that biotinylated SR-A had an approximate half-life of 1.6 (total pool of receptor) and 0.3 h (cell-surface pool of receptor) in control RAW264.7 cells (Fig. 6). NB-DNJ treatment increased the half-life of surface-biotinylated SR-A to ~ 2.3 (total) and 2 h (cell surface). IFN- γ treatment extended SR-A half-life to a comparable extent (2.4 and 2.0 h for total and cell surface-labeled populations of SR-A, respectively). The receptor half-life was further enhanced by co-treatment with NB-DNJ plus IFN- γ , to 7.9 (total) and 4.2 h (cell-surface).

DISCUSSION

Inhibiting the first steps in *N*-glycan processing, and therefore preventing the interaction of glycoproteins with calnexin, does not inevitably lead to gross protein misfolding and degradation. Instead, it can cause subtler conformational changes of certain domains of the protein. These domains would have a particular requirement for an interaction with calnexin to achieve correct regional folding. For example, the envelope glycoprotein of HIV, gp120, is heavily *N*-glycosylated and when expressed in cultured cells in the presence of glucosidase I and II inhibitors is not degraded, but there is regional misfolding of the V1 and V2 domains rendering the protein unable to undergo the post-CD4 binding conformational change required for viral fusion with the host cell (21). This effect was confirmed through the use of a panel of conformationally sensitive antibodies that detected changes only in these two regions of the protein (22). Similarly, when the key enzyme in melanin biosynthesis, tyrosinase, is synthesized in the presence of α -glucosidase inhibitors it is correctly transported to the melanosome but is not catalytically active because of an altered conformation that prevents chelation of copper into its active site (17). Subsequent site-directed mutagenesis revealed which of the conserved *N*-glycan sites were required for the protein to adopt its fully functional conformation (23). Such studies therefore help probe which domains of proteins have an absolute

FIG. 5. *A*, relative change in surface expression of SR-A on RAW264.7 cells cultured in the presence of different concentrations of NB-DNJ (open bars) or NB-DNJ plus IFN- γ (filled bars). *B*, relative change in total expression of SR-A on RAW264.7 cells cultured in the presence of 2 mM NB-DNJ (open bars) or NB-DNJ plus IFN- γ (filled bars). *C*, relative change in endocytosis of DiI-acLDL by RAW264.7 cells cultured in the presence of 2 mM NB-DNJ (open bars) or NB-DNJ plus IFN- γ (filled bars).



requirement for an interaction with calnexin and reveal the biological consequences of regional misfolding.

In this report, we describe the effects of preventing *N*-glycan processing on the biological properties of murine SR-A. This was achieved by culturing cells in the presence of the imino sugar NB-DNJ, which inhibits the endoplasmic reticulum resident processing enzymes α -glucosidases I and II, leading to the maintenance of immature glycosylated *N*-glycans (16). We observed that SR-A expressed by the untreated murine macrophage cell line RAW 264.7 interacts with calnexin and the *N*-linked oligosaccharides on the mature protein are fully processed to complex type structures. Treatment of RAW cells with NB-DNJ prevented the protein interacting with calnexin and resulted in the transport of biologically active receptors to the plasma membrane. However, the half-life of these glycosylated receptors was extended, resulting in increased SR-A expression at the cell surface and a proportional enhancement of endocytosis of the lipoprotein ligand, acLDL. SR-A, expressed by NB-DNJ-treated cells, was assembled into a mature trimeric form,

was detectable with a specific monoclonal antibody, and was functional, in terms of recognition and uptake of an endocytic ligand. This would suggest strongly that it was not grossly misfolded because of the retention of immature glycosylated *N*-glycans. It is possible that glycosylated SR-A has more subtle conformational changes that influence its cellular trafficking. Such alterations would have been undetectable with the antibody employed in this study, which binds a sequence-dependent epitope in the α -helical coil-coil domain (24). However, we cannot rule out the possibility that the increased half-life of SR-A is indirectly mediated by altered folding of other calnexin-dependent proteins or via another activity of NB-DNJ. This imino sugar also inhibits glycosphingolipid biosynthesis (25) and does so at a lower dose than that required for inhibiting *N*-glycan processing. However, at doses where complete glycolipid inhibition was achieved no effect was observed on SR-A expression, therefore excluding this as a potential mechanism affecting the rate of receptor turnover (data not shown).

Previous studies of the biochemical properties of SR-A have

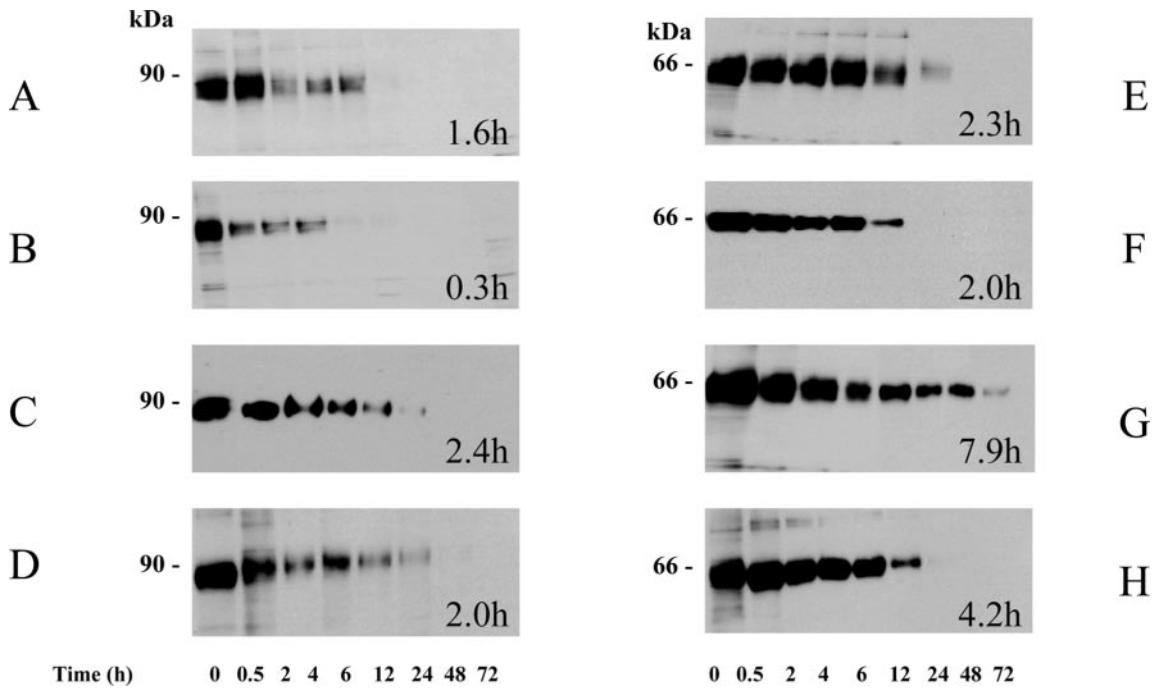


FIG. 6. Kinetics of turnover of cell surface-associated and total SR-A protein in RAW264.7 cells untreated or cultured in the presence of 2 mM NB-DNJ or 2 mM NB-DNJ plus IFN- γ . Half-life of the protein was determined by densitometry and is stated in hours (lower right-hand corner of each panel). Panel A, untreated, total expression. Panel B, untreated, cell surface expression. Panel C, +IFN- γ , total expression. Panel D, +IFN- γ , cell-surface expression. Panel E, +NB-DNJ, total expression. Panel F, +NB-DNJ, cell surface expression. Panel G, +IFN- γ and NB-DNJ, total expression. Panel H, +IFN- γ and NB-DNJ, cell surface expression.

shown that at least some of the potential *N*-linked sites are normally occupied. Bovine SR-A purified from lung tissues consisted of approximately equal amounts of reduction-sensitive trimers (220 kDa) and dimers (150 kDa) (26). Upon reduction, these oligomeric forms collapsed into monomers of about 77 kDa, ~15–20 kDa of which was because of *N*-linked glycosylation. Askenas and colleagues (9) demonstrated, through a combination of pulse labeling and subsequent *N*-glycanase digestion, that murine SR-A expressed by both the P388D1 macrophage cell line and transfected Chinese hamster ovary cells is synthesized as a glycoprotein containing *N*-linked oligosaccharide chains. The 17-kDa mass of *N*-glycanase-sensitive structures of type II SR-A (9) is in accordance with that found in this study of RAW264.7 cells (Fig. 1).

The amino acid sequence of human SR-A indicates seven potential sites for *N*-linked glycosylation of which five are conserved within three other mammalian species (Table I). These conserved sites are candidate regions of the protein that may require an interaction with calnexin to achieve correct regional folding. Studies of the behavior of single and multiple, site-specific mutant receptors should shed light on this. Two conserved sites (Asn¹⁰² and Asn¹⁴³) fall within the spacer domain, whereas the other three (Asn¹⁸⁴, Asn²⁴⁹, and Asn²⁶⁷) reside in the α -helical coiled coil domain. These two protein domains are well conserved; with the percent amino acid sequence identities across the four species being 59 and 78%, respectively (9). The biological contributions of these two extracellular protein domains as assessed by structure-function analyses have been explored to a far lesser extent than the collagenous domain and their precise biological role(s) have yet to be elucidated. Mutagenesis of the α -helical coiled coil domain has demonstrated the existence of a seven-residue sequence (Ile¹⁷³–Ser¹⁷⁹) that is required for receptor oligomerization (27) and replacement of His²⁶⁰ generated a receptor that was unable to dissociate from ligand even at acidic pH (13). Our demonstration of increased levels of functional receptor on NB-DNJ-treated cells would

indicate that specific oligosaccharide structures are not critical for oligomerization of SR-A.

We cannot exclude the possibility that the effect of the drug on intracellular trafficking of the receptor is not solely because of changes in *N*-glycosylation of SR-A itself. It is possible that its altered behavior of the receptor is also influenced to an unknown extent by the alteration of oligosaccharide chains on other molecules within the endocytic pathway. There is indirect experimental evidence that the cellular distribution of SR-A is affected by other specific cellular components. Although SR-A is expressed at high levels on the cell surface of myeloid cells, when the receptor is transfected into non-macrophage cell types, very little reaches the plasma membrane. Significant surface expression is only attained when cells are grown under conditions that pharmacologically force SR-A re-distribution (8).

The possibility that a significant part of the effect of NB-DNJ on the intracellular trafficking of SR-A is indirect would be consistent with the observation that receptor re-distribution in RAW264.7 cells was synergistically enhanced by the presence of the imino sugar together with IFN- γ . Treatment with cytokine induced an ~2-fold increase in surface expression, comparable with the imino sugar alone, whereas a 15-fold enhancement was detected when combined with NB-DNJ (Fig. 5). We hypothesize that this synergy could be explained by IFN- γ -dependent enhancement of components of the endocytic pathway that in some manner impact upon the trafficking of SR-A and whose activities are also affected by modification of their *N*-linked oligosaccharides. IFN- γ mediates many general cellular effects, including the activation of endosome proteolysis, antigen processing, and the destructive activities of the macrophage (28). In contrast its effects on specific endocytic mechanisms have been less well explored. Exposure to the cytokine has been shown to decrease the surface expression of the mannose receptor on murine J774E cells (29) and reduce both fluid phase pinocytosis and

mannose receptor-mediated uptake in human monocyte-derived macrophages (30). We were unable to investigate whether mannose receptor-dependent endocytosis was affected by NB-DNJ and IFN- γ because RAW264.7 cells do not express this receptor (31). Interestingly, whereas we found a 2-fold enhancement of SR-A expression on RAW264.7 cells with IFN- γ , Geng and Hansson (32) reported a down-regulation of receptor activity on treated human macrophages. Thus IFN- γ may display species or cell-specific effects on the expression of SR-A. In addition to affecting endocytic receptor expression, studies have demonstrated that the cytokine can also change the dynamics of membrane trafficking, which could also affect receptor turnover and recycling (33). IFN- γ activation of murine bone marrow-derived macrophages significantly slowed aspects of membrane trafficking, resulting in the delayed progression of phagosomes to endosomes and lysosomes (33). However, IFN- γ can induce the expression of specific molecular components of the endocytic pathway, such as the GTPase, Rab5a (34). The effect of the cytokine on endocytic mechanisms is clearly complex and a more detailed understanding of all its influences will require further experimentation.

A study employing a combination of biochemical and morphological approaches has given a detailed description of the endocytic pathway of SR-A and its lipoprotein ligands, in which the trans-Golgi system was identified to play a crucial role in sorting and recycling of the receptor (35). It would be of interest to use these methodologies to compare parameters of endocytosis in untreated and NB-DNJ/IFN- γ -exposed macrophages. For example, this would facilitate distinguishing between two possible explanations for the lengthened half-life of SR-A. This could be because of the extended duration of a single round of endocytosis or alternatively an increase in the numbers of rounds of recycling that receptors go through before degradation in the lysosome. We envisage that future investigations of this nature, involving molecular, cellular, and microscopic approaches should enable us to more precisely define the roles of the conserved *N*-linked oligosaccharides on SR-A and the structural protein domains that bear them, whose biological functions are currently poorly understood in comparison to the ligand-binding collagenous region. In addition, it should enable us to explore further the broader relationship between *N*-linked glycosylation, protein conformation, and endocytic mechanisms.

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