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Secretion of Glycosylation Site Mutants Can Be Rescued by the Signal/Pro Sequence of Tissue Plasminogen Activator

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Abstract Strategies that prevent the attachment of N-linked carbohydrates to nascent glycoproteins often impair intracellular transport and secretion. In the present study, we describe a method to rescue the intracellular transport and secretion of glycoproteins mutagenized to delete N-linked glycosylation sites. Site-directed mutagenesis was used to delete N-linked glycosylation sites from a chimeric protein, TNFR-IgG1. Deletion of any of the three glycosylation sites in the TNFR portion of the molecule, alone or in combination, resulted in a moderate or near total blockade of TNFR-IgG1 intracellular transport and secretion. Pulse chase experiments suggested that the glycosylation site mutants accumulated in the endoplasmic reticulum (ER) and were inefficiently exported to the Golgi apparatus (GA). Replacement of the TNFR signal sequence with the signal/pro sequence of human tissue plasminogen activator (tPA) overcame the blockade to intracellular transport, and restored secretion to levels comparable to those achieved with the fully glycosylated molecule. Ligand binding studies suggested that the secreted glycosylation variants possessed binding characteristics similar to the fully glycosylated protein. This study demonstrates that N-terminal sequences of tPA are unexpectedly efficient in facilitating transport from the ER to the GA and suggests that these sequences contain a previously unrecognized structural element that promotes intracellular transport. J. Cell. Biochem. 75:446–461, 1999.

Key words: glycosylation; signal sequence; secretion; TNFR; TNFR-IgG1

Many eukaryotic cell surface and secreted proteins are post-translationally processed to incorporate N-linked and O-linked carbohydrates [Kornfeld and Kornfeld et al., 1985; Rademacher et al., 1988]. Protein glycosylation is thought to subserve a variety of functions including: augmentation of protein folding, inhibition of protein aggregation, regulation of intracellular trafficking to organelles, modulation of sensitivity/resistance to proteolysis, modulation of protein antigenicity, and signal transduction [Alexander and Elder et al., 1984; Caton et al., 1982; Fiedler and Simons, 1995; Flack et al., 1994; Helenius, 1994; Olden et al., 1978]. In higher organisms, the nature and extent of glycosylation can markedly affect the circulating half-life and bioavailability of secreted proteins by mechanisms involving receptor-mediated uptake and clearance [Ashwell and Harford, 1982; Ashwell and Morrell, 1974]. Thus, several receptor systems have been identified that are thought to play a major role in the clearance of serum proteins through recognition of incompletely sialated glycoproteins [Kery et al., 1992; Stockert, 1995].

In the development of recombinant glycoproteins for use as pharmaceutical products, it has long been speculated [Berman and Lasky et al., 1985] that the pharmacodynamics of recombi-

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nant proteins could be modulated by the addition or deletion of glycosylation sites. Thus, expression strategies resulting in incomplete attachment of terminal sialic acid residues to oligosaccharide terminii might provide a means of shortening the bioavailability and half-life of secreted glycoproteins by promoting clearance by the hepatic asialoglycoprotein or mannose receptors. Conversely, expression strategies resulting in saturation of oligosaccharide terminii with sialic acid might increase protein bioavailability and half-life by preventing uptake by the hepatic receptors. An alternative strategy to prevent uptake and clearance by glycoprotein receptors is the deletion of glycosylation sites altogether.

However, many studies [Brunner et al., 1992; Collier et al., 1993; Dash et al., 1994; Gallagher et al., 1988; Kobayashi et al., 1994; Leavitt et al., 1977; Machamer and Rose, 1988] have reported that the deletion of N-linked glycosylation sites often impairs intracellular transport and results in the intracellular accumulation of glycosylation variants. Endoglycosidase sensitivity and immunofluorescence studies suggest that glycosylation variants are retained in the ER and cannot be transported to the GA. Thus, while glycosylation site variants of secreted proteins can be expressed intracellularly, it has proven difficult to recover useful quantities from growth-conditioned cell culture medium.

In this paper, we have investigated the possibility that the retention of glycosylation mutants in the endoplasmic reticulum might be overcome by altering N-terminal sequences including and/or adjacent to the signal sequence. Previously, we reported [Berman et al., 1989; Lasky et al., 1986] that transfection of the HIV-1 *env* gene fragment encoding gp120 into CHO cells resulted in abundant protein synthesis, but that glycoprotein export and secretion were defective, resulting in the accumulation of nascent protein in the ER. These studies showed that the block to secretion could be partially overcome by replacing the signal sequence of gp120 with an N-terminal sequence of herpes simplex virus glycoprotein D (gD) [Berman et al., 1989; Lasky et al., 1986].

In the present study, we systematically deleted the four glycosylation sites in TNFR-IgG1 and compared the secretion efficiency of the resulting glycosylation variants. TNFR-IgG1 is a chimeric protein created by fusion of the extracellular domain of the 55 kilodalton tumor necrosis factor receptor (TNFR) fused to the Fc and hinge region immunoglobulin G_1 (IgG_1) [Ashkenazi et al., 1991]. As expected from previous studies, we observed that the intracellular transport of most, but not all, of the TNFR-IgG1 glycosylation mutants was severely impaired. We reasoned, however, that the barrier to intracellular transport of TNFR-IgG1 might be overcome by altering the N-terminal sequences, including the signal sequence. The results described demonstrate that replacement of the signal sequence of TNFR with N-terminal sequences of human tissue plasminogen activator (tPA) can markedly facilitate export from the ER to the GA and greatly improve the secretion efficiency of proteins mutagenized to remove N-linked glycosylation sites.

METHODS

DNA and Constructions

The chimeric gene encoding TNFR-IgG1 [Ashkenazi et al., 1991] was kindly provided by A. Ashkenazi (Genentech, Inc.). The gene encoding tissue-type plasminogen activator (tPA) [Pennica et al., 1983] was kindly provided by C. Crowley (Genentech, Inc.). Replacement of the TNFR signal sequence with the tPA signal/pro sequence was accomplished using site-directed mutagenesis [Kunkel, 1985] to insert a BglII restriction site between the signal sequence and the mature start of the TNFR-IgG1. The tPA signal/pro sequence fragment (35 amino acids) was amplified by PCR using a 5′primer containing an EcoRI site and a 3′primer containing an in-frame BglII site. The tPA EcoRI- BglII fragment was then ligated into the TNFR-IgG1 containing plasmid cut with BglII and EcoRI to create pRK5-tPA-TNFR-IgG1.

Deletion of glycosylation sites was carried out by directed mutagenesis using a mismatched primer method [Kunkel, 1985] using the Muta-Gene M13 Kit (Bio-Rad Inc., Hercules, CA). Mutations were verified by dye terminator cycle sequencing using the automated ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). For simplicity, the glycosylation mutants described in this paper are named using the single letter amino acid code with reference to the amino acid residue present at each of the four N-linked glycosylation sites at amino acid positions 14, 105, 111, and 248 (Fig. 1A). Thus, the designation NNNN represents the fully glycosylated TNFR-IgG1, whereas NQNN indicates TNFR-IgG1 where glutamine

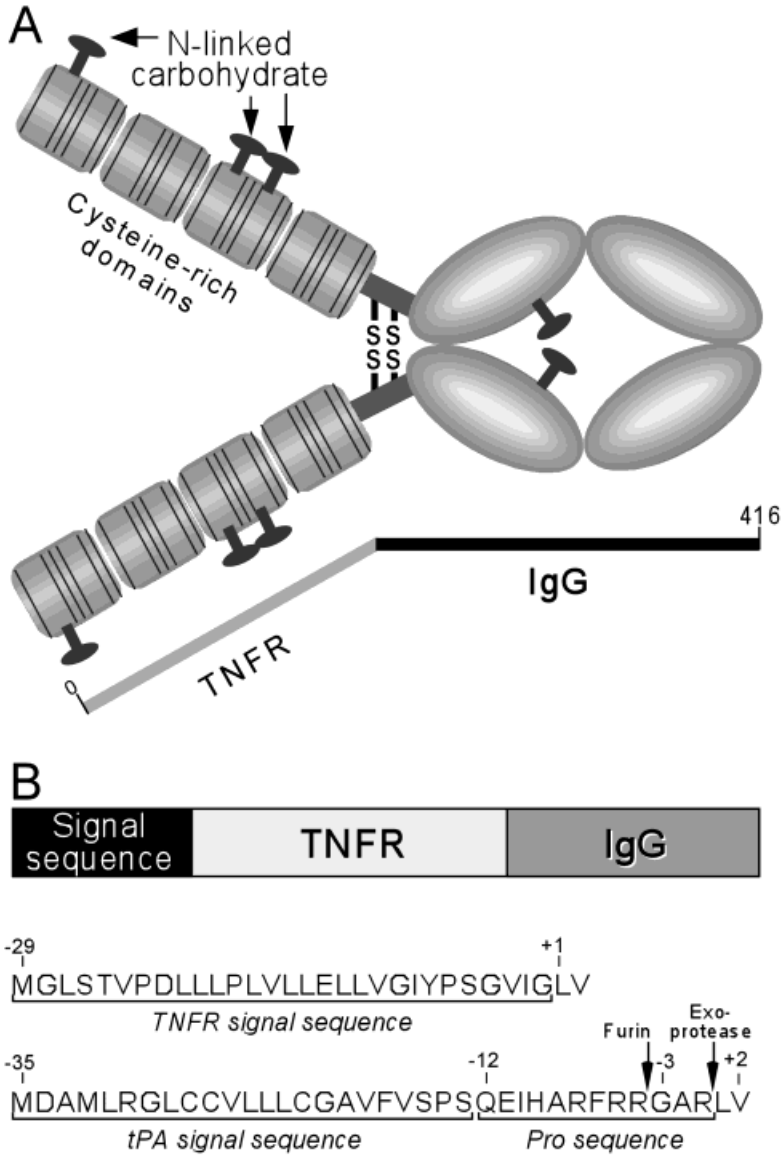

Fig. 1. Diagram of TNFR-IgG1 and signal sequences. TNFR-IgG1 is a chimeric protein consisting of the extracellular domain of the TNF receptor fused to the hinge and Fc domain of immunoglobulin heavy chain. TNFR-IgG1 is secreted as a homodimer with four N-linked glycosylation sites per monomer. The proteins expressed in this study were synthesized using the wild-type TNFR signal sequence containing 30 amino acids, or the signal and pro-sequence of human tissue plasminogen activator (tPA) containing 35 amino acids.

replaced asparagine at the second glycosylation site (position 105). TNFR-IgG1 glycosylation variants were cloned into the expression vector pRK5 [Eaton et al., 1986] that enabled transient expression in the human embryonic kidney 293 cell line. The pRK5 chimeric transcription unit includes a cytomegalovirus (CMV) immediate early promoter and a simian virus 40 (SV-40) polyadenylation site. For expression in CHO cells, genes encoding TNFR-IgG1 variants were cloned into the closely related expression vector pSVI6B5 that differs from pRK5 in that transcription is directed by SV-40 promoter and enhancer elements rather than by the CMV promoter.

Cells and Transfections

293s human embryonic kidney (HEK) cells were cultured in 100 mm culture plates containing a mixture of Dulbecco's modified Eagles medium (DMEM) and Ham's F12 medium (F12) (Gibco BRL, Grand Island, NY) supplemented with 10% whole fetal bovine serum (FBS) and incubated at 37°C in an atmosphere containing 5% CO_2. Confluent plates of 293 cells were passaged at a ratio of 1:5 and were transfected at 60% confluency with plasmids containing TNFR-IgG1 glycosylation variants by the calcium phosphate method [Graham and Van der Eb et al., 1973]. For expression of TNFR-IgG1

variants in CHO cell lines, the genes encoding TNFR-IgG1 glycosylation site mutants were cloned into the pSVI6B5 expression vector. These plasmids were then co-transfected into CHO cells deficient in the production of dihydrofolate reductase (dhfr) along with a plasmid (pFD11) containing a cDNA encoding murine dhfr. The transfected cells were selected for the ability to grow in culture medium deficient in glycine, hypoxanthine, and thymidine. Resulting colonies were picked and then selected for growth in varying concentrations of methotrexate. Stable cell lines expressing TNFR-IgG1 glycosylation site were scaled up to 10 L in 15 L bioreactors, and growth-conditioned cell culture medium was harvested for affinity purification (described below).

Metabolic Labeling and Immunoprecipitation

Two days post-transfection, the culture medium was removed from the transfected 293 cells and the cell monolayer washed twice with phosphate buffered saline (PBS). Cells were incubated in methionine- and cysteine-free DMEM and supplemented with ProMix ^{35}S-Cell Labeling Mix (Amersham, Arlington Hts, IL) (100 μCi/mL). The cells were labeled 8–16 h at 37°C in an atmosphere of 5% CO_2. After labeling, the cell supernatants were removed, centrifuged at 4,000 rpm for 5 min, and 1 mL was aliquoted for immunoprecipitation experiments. The labeled cells were washed three times with PBS, lysed directly on the culture dishes with cell lysis buffer (PBS containing 3% NP-40), and centrifuged at 14,000*g* for 5 min. The lysate was transferred to a fresh tube, and 200 μL was removed for immunoprecipitation. Immunoprecipitation of both supernatants and lysates was accomplished by addition of 30 μL of *Staphylococcus aureus* Protein A Sepharose (Pharmacia Inc., Piscataway, NJ). After a short incubation period on ice (10–15 min) the Protein A Sepharose/TNFR-IgG1 complexes were sedimented by centrifugation at 14,000*g* for 1 min, washed in wash buffer (PBS, 1% NP-40, 0.1% sodium dodecyl sulfate; SDS), and resuspended in 2 × sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 5% beta-mercaptoethanol (2-ME). The immunoprecipitated proteins were resolved by SDS-PAGE using 10% Tris-Glycine polyacrylamide gels (Novex, Inc., San Diego, CA). Proteins were visualized by autoradiography and mobilities were calculated with reference to ^{14}C-Methylated Rainbow Colored Protein Molecular Weight Markers (Amersham). Proteins were quantitated by densitometric scanning of autoradiographs using the Personal Densitometer (Molecular Dynamics, Sunnyvale, CA).

A listing of all the mutants analyzed and a qualitative assessment of secretion efficiency compared to wild-type TNFR-IgG1 are provided in Table I.

Pulse Chase Analysis

Forty-eight hours post-transfection, cells were pulse labeled with ProMix ^{35}S-Cell Labeling Mix (500 μCi /mL) for 15 min. The labeling medium was then removed and the cell monolayer was washed at room temperature with PBS. Fresh medium (DMEM/F12 + 10% FBS) was then added, and cells were harvested at various time-points. For harvesting, plates were chilled on ice; supernatants were removed, and the cell monolayer was washed once with PBS. Cells were then lysed with 1 mL of lysis buffer. Lysate and supernatant samples were aliquoted and immunoprecipitated as described above.

TABLE I. Summary of TNFR1-IgG1 Glycosylation Mutants

Amino acid position				Secretion efficiency
14	105	111	248	
N	N	N	N	+++
Q	N	N	N	++
N	Q	Q	N	+
N	N	N	Q	+++
N	N	Q	N	+/−
N	Q	N	N	−
Q	N	N	Q	+
N	N	Q	Q	+
Q	Q	Q	N	+/−
Q	Q	Q	Q	−
N	S	N	N	+++
N	N	S	N	++
N	D	D	N	+
N	K	K	N	++
N	S	S	N	−
N	R	R	N	+
N	T	T	N	+/−
Q	S	N	Q	−
N	D	D	Q	+
N	K	K	Q	++

Endoglycosidase Digestion

Endoglycosidase H (endo H) (1 mU/μl) was purchased from Boehringer-Mannheim (Indianapolis, IN). The metabolic labeling and the following immunoprecipitation was done as described above. The Protein A Sepharose pellets were resuspended in 1.25% Triton X-100, endoglycosidase digest buffer (50 mM Na citrate, pH 5.5) and 2 μl of the Endoglycosidase H enzyme. Samples were incubated for 12–16 h at 37°C. After the endoglycosidase H digestion, the immunoprecipitated sepharose was pelleted by centrifugation and the protein extracted from the sepharose in SDS-PAGE sample buffer containing beta-mercaptoethanol (BME). The proteins were resolved by SDS-PAGE using 10% Tris-Glycine polyacrylamide gels (Novex). Proteins were visualized by autoradiography and mobilities were calculated with reference to ^{14}C-Methylated Rainbow Colored Protein Molecular Weight Markers (Amersham).

Purification of TNFR-IgG1 Glycosylation Site Mutants

Stable CHO cultures expressing TNFR-IgG1 glycosylation mutations were scaled up to 10 L in a 15 L bioreactor. Secreted TNFR-IgG was purified from conditioned culture media by immobilized *S. aureus* protein A affinity chromatography via a modification of a method described previously [Chamow et al., 1994]. Four TNFR-IgG1 variants were purified: NNNN, NNQQ, NSNQ, and QSNQ. Briefly, purification was accomplished by a four step process. (1) Prior to sample loading, the Protein A column was equilibrated with 20 mM Tris buffer, 150 mM NaCl (pH 7.4). (2) After the growth-conditioned cell culture medium was loaded onto the column, the column was sequentially washed with the following buffers: 20 mM Tris buffer, 150 mM NaCl (pH 7.4); 20 mM Tris buffer 500 mM TMAC (pH 7.4); and 20 mM Tris buffer (pH 7.4). (3) The glycosylation site mutants were eluted with 50 mM citric acid, 20% (w/v) glycerol (pH 3.0) and the elution pools were subsequently adjusted to pH 6.0 using 1.0 M sodium citrate. Finally, (4) the eluates were buffer exchanged by gel filtration chromatography into PBS (pH 7.4). The purified mutants were analyzed using SDS-PAGE (12.5%) with Coomassie blue staining.

TNF Binding Assay

The binding of TNF to TNFR-IgG1 and TNFR-IgG1 glycosylation site mutants was determined using a competitive binding immunoassay similar to that described by Ashkenazi et al. [1991]. Briefly, microtiter plates were coated with affinity-purified goat antibodies to human IgG Fc domain. Purified TNFR-IgG1 or TNFR-IgG1 glycosylation site mutants were captured on the plates and then reacted with varying concentrations of unlabeled TNF (0.8 to 800 nM) and a fixed amount of (^{125}I)-labeled TNF (0.05 nM). After washing the amount of TNF remaining bound to the wells of the microtiter plates was determined with a gamma counter. Data were fit using a four parameter curve. The effective concentration of unlabeled TNF that resulted in half maximal (50%) binding of (^{125}I)-labeled TNF to TNFR-IgG1 glycosylation mutants was reported as EC_{50} values.

RESULTS

TNFR-IgG1 is a chimeric protein constructed by fusing the extracellular domain of the receptor for TNF alpha with sequences encoding the Fc domain and hinge region of IgG1 [Ashkenazi et al., 1991]. The chimeric protein (Fig. 1A) contains four N-linked glycosylation sites: three located in the TNFR region (positions 14, 105, and 111) and one in the Fc domain (position 248). Previous studies have shown that TNFR-IgG1 is secreted as a homodimer, binds to TNF alpha with high affinity, and has potent anti-inflammatory activity in vivo [Ashkenazi et al., 1991].

Pulse chase experiments were carried out to characterize the secretion efficiency of TNFR-IgG1. In these studies, 293 cells were transiently transfected with a calcium phosphate precipitated plasmid (pRK.TNFR-IgG1) and cultured for 2 days. The cells were pulse labeled with (^{35}S)-methionine, and samples were collected at various time points. Immunoprecipitation studies (Fig. 2) indicated that TNFR-IgG1 first appeared in the cell culture medium approximately 1 h after pulse labeling, and that the amount of secreted protein progressively increased over the entire 24-h observation period. These studies also showed that a significant fraction of the TNFR-IgG1 synthesized during a 15-min pulse labeling was retained inside the cells. Densitometric analysis of these data (Fig. 3A) demonstrated that only about

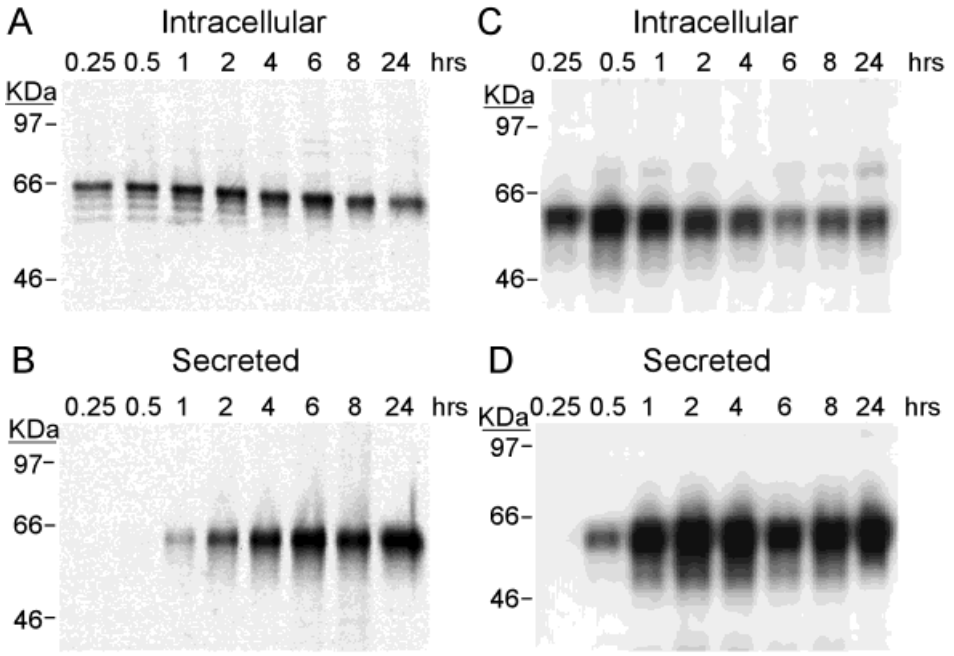

Fig. 2. Effect of the signal sequence on the secretion kinetics of TNFR-IgG1 and tPA.TNFR-IgG1. Parallel cultures of human embryonic kidney cells (293s) were transfected with plasmids encoding either TNFR-IgG1 or tPA.TNFR-IgG1 for pulse labeling experiments. Two days post-transfection, the culture medium was removed and replaced (after washing two times) with methionine- and cysteine-free DMEM supplemented with (^{35}S)-labeled methionine and cysteine. The cells were labeled at 37°C (5% CO_2) for a 15-min pulse and the plates were then washed twice with PBS and returned to the incubator. Plates were then harvested at the times indicated. The labeling reaction was terminated by washing the cells immediately in chilled (4°C) PBS followed by detergent extraction. Cell lysates and cell culture supernatants were precipitated by the addition of Protein A Sepharose. The Protein A Sepharose:TNFR-IgG1 complexes were pelleted by centrifugation, washed repeatedly, and eluted in SDS-PAGE sample buffer containing beta-mercaptoethanol. The eluted protein was resolved on 10% SDS-PAGE gels and visualized by autoradiography. **A:** Intracellular (i.e., cell associated) TNFR-IgG1. **B:** Secreted TNFR-IgG1. **C:** Intracellular tPA.TNFR.IgG. **D:** Secreted tPA,TNFR.IgG.

50% of the pulse-labeled protein was secreted from the cell during a 24-h period, and that labeled precursor appeared to remain in a stable intracellular pool for more than 24 h. The kinetics and pattern of secretion observed for TNFR-IgG1 were similar to those previously reported for two other secreted variants of membrane glycoproteins [Berman, 1985; Berman et al., 1989] suggesting that secretion of TNFR-IgG1 was similarly inefficient.

Endoglycosidase H digestion (Fig. 4) studies, similar to those described by Machamer and Rose [1988], demonstrated that cell-associated TNFR-IgG1 was sensitive to endo H digestion, whereas secreted TNFR-IgG1 was resistant to endo H digestion. These results suggested that the intracellular TNFR-IgG1 possessed the high mannose form of N-linked carbohydrates characteristic of proteins retained in the ER, whereas the secreted TNFR-IgG1 acquired the complex, sialic acid-containing form of N-linked carbohydrates acquired during transit through the Golgi apparatus. An ER localization for intracellular TNFR-IgG1 was further suggested by confocal immunofluorescence studies [Köhne et al., manuscript in preparation] where intracellular TNFR-IgG1 co-localized with two ER resident proteins immunoglobulin heavy chain binding protein (BIP) and protein disulfide isomerase (PDI), and could not be detected on the cell surface.

Deletion of Glycosylation Sites Can Impair Secretion of TNFR-IgG1

To examine the effect of sequential deletion of glycosylation sites on the secretion of TNFR-IgG1, further experiments were carried out with a series of glycosylation mutants (Table I). Secretion efficiency was estimated by scanning densitometry where the amount of secreted protein was compared to the total protein immunoprecipitated from lysate and cell culture medium. In initial studies, mutagenesis primers were designed to replace the codon specifying

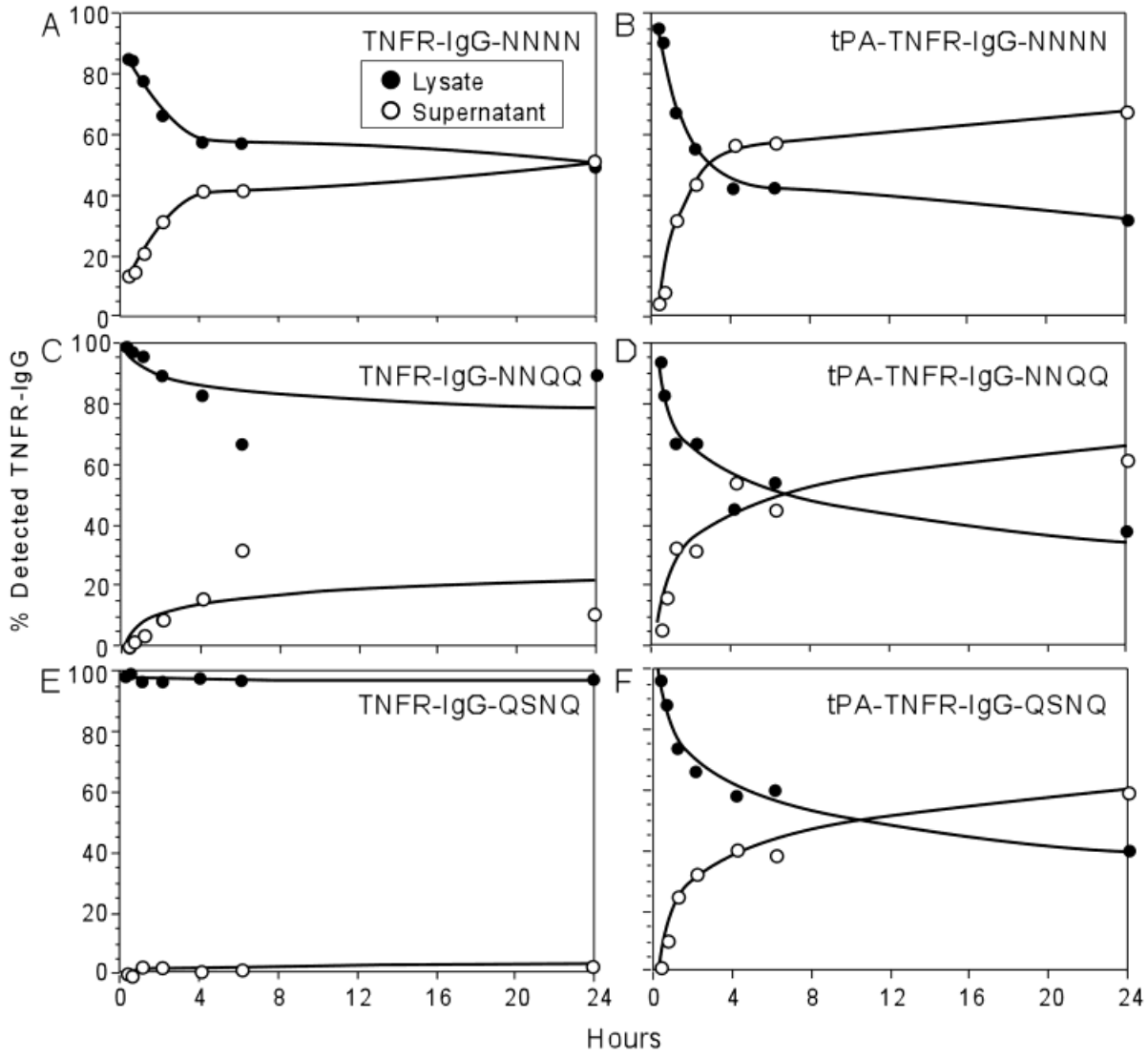

Fig. 3. Secretion kinetics of TNFR-IgG1 signal sequence and glycosylation mutants. Plasmids encoding fully glycosylated (A) or mutated forms of TNFR-IgG1 (B-F) were transfected into 293s cells and analyzed by pulse-chase analysis as described in Figure 2. Autoradiographs were analyzed with a scanning densitometer and the optical density values for supernatants (o) and cell lysates (●) were normalized and plotted as a function of time (0–24 h). **A:** TNFR-IgG1; **B:** tPA.TNFR-IgG1; **C:** NNQQ; **D:** tPA.NNQQ; **E:** QSNQ; **F:** tPA.QSNQ.

asparagine (N) in the N-linked carbohydrate attachment sequon, asparagine-X-serine/threonine (N-X-S/T), with a codon specifying glutamine (Q). Because glycosylation sites 2 and 3 in TNFR-IgG1 are only a few amino acids apart (residues 105 and 111), a single mutagensis primer could be used to mutate both sites simultaneously. We found that all of the glycosylation mutants were expressed, but there were significant differences in secretion efficiencies (Fig. 5A). High levels of background binding to cellular proteins were seen in some cell lysates. Removal of the fourth glycosylation site (residue 248), located in the Fc domain of TNFR-IgG1 (NNNQ) had no effect on secretion relative to wild-type TNFR-IgG1 (NNNN). In contrast, removal of glycosylation sites 2 and 3 (NQQN) resulted in a nearly complete blockade of secretion. Deletion of the first glycosylation site (QNNN) also blocked secretion; however, in this case only about 20% of nascent protein was secreted compared to 50% of total protein (intracellular and secreted) as seen with the fully glycosylated protein. Removal of the first three

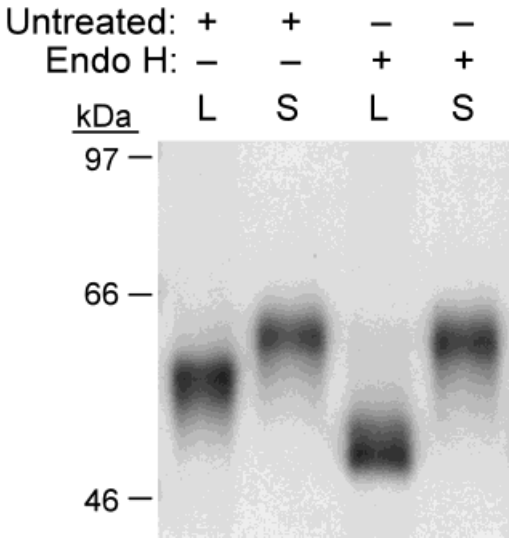

Fig. 4. Intracellular Endo H sensitivity of the TNFR-IgG1 molecule. 293s cells were transfected with the TNFR-IgG1 plasmid via calcium phosphate precipitation. Two days post-transfection, the culture medium was removed and replaced (after washing two times) with methionine- and cysteine-free DMEM supplemented with (^{35}S)-labeled methionine and cysteine. Cells were labeled overnight at 37°C (5% CO_2). Cell lysates and cell culture supernatants were precipitated by the addition of Protein A Sepharose. The Protein A Sepharose:TNFR-IgG1 complexes were pelleted by centifugation, washed repeatedly . One lysate and supernatant sample was treated with Endo H, the other one was treated with endoglycosidase digest buffer. Samples were eluted in SDS-PAGE sample buffer containing beta-mercaptoethanol and the eluted protein was resolved on 10% SDS-PAGE gels. The mobilities of molecular weight markers are indicated at the left margins. The untreated lysate and supernatant samples were loaded in **lanes 1** and **2**, **lane 3** contained the endo H sensitive lysate, whereas the endo H resistant supernatant sample was loaded in **lane 4**.

glycosylation sites (QQQN) completely inhibited secretion as did removal of all four glycosylation sites (QQQQ).

Further mutants were constructed (Table I) to study, independently, the influence of glycosylation sites 2 and 3 on secretion. It was found that removal of glycosylation site 3 alone (NNQN) allowed approximately 30% nascent protein to be secreted whereas, deletion of glycosylation site 2 alone (NQNN) completely abolished secretion (Fig. 5B). Deletion of glycosylation sites 3 and 4 (NNQQ) resulted in a low secretion efficiency (Fig. 5C) (approximately 50% secretion of nascent protein) that was similar to the NNQN mutant (approximately 30%) but lower than the NNNQ mutant (approximately 20%) (Fig. 5A). Deletion of glycosylation sites 1 and 4 (QNNQ) resulted in a secretion level (Fig. 5C) that was similar to the QNNN mutant, though distinctly lower than the NNNQ mutant (Fig. 5A). These studies suggest that mutations in the TNFR domain can impair secretion efficiency and are not effected by the presence or absence of N-linked carbohydrate in the Fc domain.

Nature of the Amino Side Chain Used to Mutate N-Linked Glycosylation Site Can Affect Secretion Efficiency

Additional studies were carried out to determine if the nature of the amino acid side chain used for the replacement of the N residue in the sequon creating the N-linked glycosylation site, affects secretion efficiency. Each N-linked oligosaccharide chain can possess up to four sialic acid residues, depending on the antennarity. These charged sialic acid residues can potentially increase solubility of the TNFR-IgG1 molecule. We reasoned that replacement of N at N-linked glycosylation sites with charged residues (e.g., aspartic acid, glutamic acid, lysine, arginine) might have less effect on conformation than replacement with hydrophilic uncharged side-chains (e.g., glutamine, threonine, serine). We found that replacement of the N residues at glycosylation sites 2 and 3 with acidic, aspartic acid residues (NDDN) or basic, lysine residues (NKKN) allowed for better secretion (Fig. 5B) than the uncharged glutamine residues in the (NQQN) construct (Fig. 5A); however, the amount of protein secreted was less (approximately 20%) than that observed with the wild-type TNFR-IgG1 protein. Replacement of asparagine at sites 2 and 3 with arginine (NRRN), also gave better secretion than the NQQN mutant, but the total amount of secreted protein appeared to be less compared to the NDDN or NKKN mutants (Fig. 5D).

We next considered the effect of uncharged, hydrophilic side chains of serine and threonine on the secretion efficiency of TNFR-IgG1. We found that single substitutions of serine for asparagine at glycosylation sites 2 and 3 gave somewhat different results (Fig. 5D). The replacement of serine for asparagine at glycosylation site 2 (NSNN) allowed for better secretion (approximately 30% of total protein) than any of the other amino acid replacements examined at this position. In contrast, replacement of serine for asparagine at glycosylation site 3 (NNSN) allowed for some secretion, but it was not as good as the NSNN (Fig. 5D). Surpris-

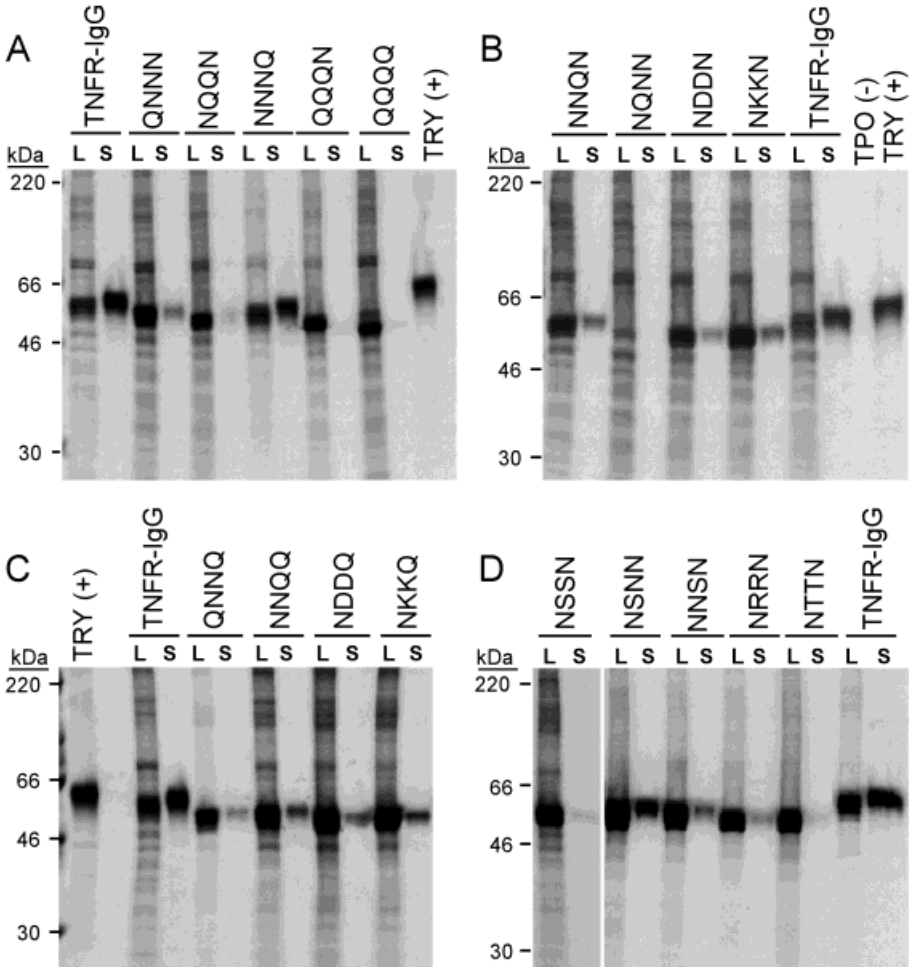

Fig. 5. Immunoprecipitation of TNFR-IgG1 glycosylation mutants. Plasmids encoding either TNFR-IgG1 glycosylation site mutants were transfected into 293s cells using calcium phosphate precipitated DNA. Two days post-transfection the culture medium was removed and replaced (after washing two times) with methionine- and cysteine-free DMEM supplemented with (^{35}S)-labeled methionine and cysteine. Cells were labeled overnight at 37°C (5% CO_2). Cell lysates and cell culture supernatants were precipitated by the addition of Protein A Sepharose. The Protein A Sepharose/TNFR-IgG1 complexes were pelleted by centifugation, washed repeatedly and eluted in SDS-PAGE sample buffer containing beta-mercaptoethanol. The eluted protein was resolved on 10% SDS-PAGE gels and visualized by autoradiography. The glycosylation site mutants are identified using the single letter amino acid code with reference to the amino acid residue present at each of the four N-linked glycosylation sites (amino acid positions 14, 105, 111, and 248). Thus, the designation NNNN represents the wild-type TNFR-IgG1, whereas NQNN indicates TNFR-IgG1 where glutamine replaced asparagine at the second glycosylation site (position 105). The glycosylation site mutants were examined in four separate experiments (**A–D**) where transiently expressed TNFR-IgG1 (TNFR-IgG1) expressed in 293s cells or a stable CHO cell line (TRY+) was used as positive controls. Background binding was determined in experiments with cells transfected with thrombopoeitin (TPO-). The mobilities of molecular weight markers are indicated at the left margins.

ingly, replacement of glycosylation sites 2 and 3 with serine (NSSN) or threonine (NTTN) completely inhibited secretion (Fig. 5D). Thus, deletion of the two adjacent glycosylation sites with either S or T, markedly inhibited secretion, whereas mutation of either site independently allowed for moderate secretion efficiency. Another construct with two glycosylation sites deleted was created by replacing N with S at site 2, and N with Q at site 4, generating NSNQ (Fig. 6B). This variant exhibited good secretion efficiency and showed that deletion of the fourth glycoslyation site did not negatively effect secretion relative to the NSNN mutant (Fig. 5D). Our results gave no indication why secretion was preserved when serine replaced asparagine at glycosylation sites 2 or 3. Although there is a theoretical possibility that either of

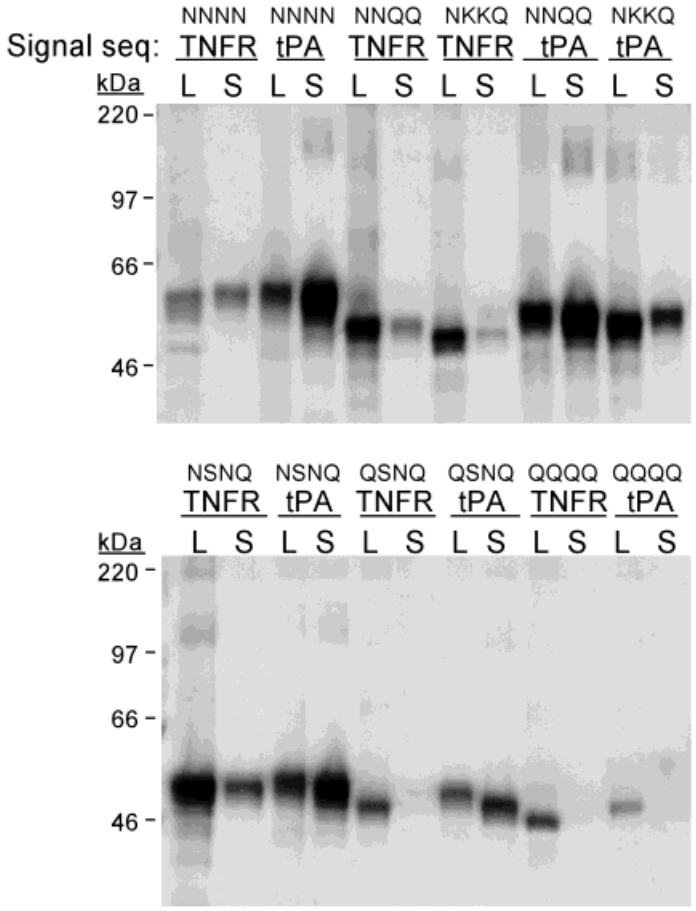

Fig. 6. Effect of signal sequence replacement on the secretion of TNFR-IgG1 glycosylation site mutants. Plasmids encoding TNFR-IgG1 glycosylation site mutants containing the tPA signal/pro sequence (tPA) or the TNFR signal sequence (TNFR) were transfected into 293s cells, metabolically labeled with (^{35}S) methionine and cysteine, and immunoprecipitated from cell culture supernatants (S) or cell lysates (L) by the addition of Protein A Sepharose as described in Figure 4. The Protein A Sepharose/TNFR-IgG1 complexes were pelleted by centrifugation, resolved by SDS-PAGE, and visualized by autoradiography. The glycosylation site mutants are identified using the single letter amino acid code with reference to the amino acid residue present at each of the four N-linked glycosylation sites. The mobilities of molecular weight markers are indicated at the left margins.

these replacements might create an O-linked glycosylation site, we have no data to support this possibility.

Triple Deletion Mutants

To investigate the effect of glycosylation site 4 in combination with sites 1–3, a series of mutants were constructed where three of the four glycosylation sites were deleted (Table I). It was found that the NDDQ, NKKQ, NDDN, and NKKN mutants were all secreted from transfected cells (Fig. 5B,C). However, in all four cases 20% less of the total protein was secreted . Thus, four constructions were identified that allowed some secretion with three of the four glycosylation sites deleted. A final mutant where three of the four glycosylation sites were deleted (QSNQ) was also examined (Table I, Fig. 6). This variant accumulated intracellularly and was not secreted.

Secretion Efficiency of TNFR-IgG1 Can Be Improved by Altering the Signal Sequence

Previous studies (Brousseau and Etcheverry, Genentech, Inc., personal communication) suggested that the yield and secretion efficiency of TNFR-IgG1 from stably transfected CHO cells could be improved by replacement of the TNFR-IgG1 signal sequence with that of human tissue plasminogen activator (tPA). To determine whether the tPA signal/pro sequence improved yield by increasing the efficiency of intracellular transport, a plasmid (pRK.tPA-TNFR-IgG1) was constructed for transient transfection studies in 293s HEK cells. Pulse chase studies (Figs. 2C,D, 3B) showed that replacement of the TNFR signal sequence with the tPA signal/pro sequence resulted in a significant improvement in secretion efficiency. Thus, after 24 h approximately 70–80% of the pulse-labeled TNFR-IgG1 was secreted into the cell culture medium when the tPA signal/pro sequence was in-

cluded, whereas only 40–50% of the protein was secreted using the wild-type TNFR signal sequence. The increased efficiency of secretion achieved with the tPA signal/pro sequence was apparent from the kinetics of secretion of TNFR-IgG1 (Figs. 2, 3A,B). When the wild-type TNFR signal sequence was used, only 40% of the pulse labeled TNFR-IgG1 was secreted in 2–4 h after pulse labeling. However, when the TNFR signal sequence was replaced with the tPA signal/pro sequence, approximately 60% of the synthesized TNFR-IgG1 was secreted in this time frame.

Studies of the biosynthesis of tPA [Berg and Grinnell, 1991] have shown that the N-terminal processing of tPA is complex and involves co-translational cleavage of a 23 amino acid signal sequence, post-translational cleavage of a 12 amino acid pro-sequence at a furin cleavage site with an extracellular cleavage of a 3 amino acid N-terminal peptide by an undefined exopeptidase (Fig. 1B). Since furin is known to be localized in the trans-GA [Berg and Grinnell, 1991; Steiner et al., 1992], the pro-sequence would not be expected to be removed until the tPA-TNFR-IgG1 precursor has been transported from the ER to the trans-GA. This complex pattern of post-translational processing appears to account for differences in electrophoretic mobilities seen in cell lysates between TNFR-IgG1 variants containing the tPA signal/pro sequence compared to those containing the wild-type TNFR signal sequence (Fig. 6). As expected from the analysis by Berg and Grinnell [1991], protein immunoprecipitates from cell lysates containing the tPA signal/pro-sequence run slower on SDS-PAGE gels than native TNFR-IgG1, suggesting that the protein accumulates intracellularly with the pro-sequence still attached. Similarly, the mobilities of the intracellular forms of the QSNQ, NSNQ, NNQQ, and NKKQ mutants containing the tPA signal/pro sequence are all slower than the mobilities of the corresponding variants containing the TNFR signal sequence (Fig. 6). However, the difference in molecular weights between the proteins expressed with the tPA signal/pro sequence and the TNFR signal sequence disappears when the secreted, extracellular forms of the proteins are examined (Fig. 6), suggesting that the tPA pro-sequence was removed after export from the ER during transit to the cell surface. The results obtained are consistent with an accumulation of TNFR-IgG1 variants in the ER, followed by a rapid, stochastic export through the Golgi Apparatus to the cell surface. Since the furin endoprotease is restricted to the trans-Golgi compartment, it is likely that the tPA pro-sequence is removed at this location.

Secretion Efficiency of Glycosylation Site Mutants Can Be Improved by Altering the Signal Sequence

To determine whether signal sequence exchange could improve the secretion efficiency of the TNFR-IgG1 glycosylation site mutants described in Figure 5, the TNFR signal sequence was deleted and replaced by the tPA signal/pro sequence. It was found (Table II, Fig. 6) that the signal sequence exchange resulted in a marked increase in secretion efficiency of these glycosylation site mutants. For example, only about 20% of the NNQQ mutant was secreted using the TNFR signal sequence, whereas 60–70% of the NNQQ mutant containing the tPA signal/pro sequence was secreted (Figures 3C and D, Figure 6). Similarly, little or none of the QSNQ mutant was secreted containing the TNFR signal sequence, but approximately 60% was secreted from the tPA signal/pro sequence containing variant (Figs.3E,F, 6). Similar improvements in secretion efficiency were observed for the NKKQ and NSNQ mutants (Fig. 6). Densitometric analysis of pulse chase experiments (Fig. 3) showed that attachment of the tPA signal/pro sequence accelerated the kinetics of intracellular transport as well as increasing the total amount of secreted protein. Although the replacement of the wild-type TNFR signal se-

TABLE II. Effect of tPA Signal Sequence or Secretion of TNFR1-IgG1 Glycosylation Mutants

Mutant	% Secretion efficiency[a]		EC_{50} for TNF binding (nM)[b]
	TNFR.ss	tPA.ss	
N N N N	50	70	6.66 ± 0.73
N N Q Q	20	70	4.74 ± 0.48
N S N Q	<5	60	6.94 ± 0.83
N K K Q	10	40	ND
Q S N Q	<5	65	2.34 ± 0.22
Q Q Q Q	<5	<5	ND

[a]Data represent results from pulse chase experiments (e.g., Fig. 3) where the percentage of pulse labeled protein secreted in a 24-h period was measured by scanning densitometry.

[b]Data represent EC_{50} values for the displacement of (^{125}I)-labeled TNF by TNFR-IgG1 glycosylation site mutants (Fig. 7).

quence with the tPA signal/pro sequence could overcome the blockade of protein secretion for many of the glycosylation mutants, this strategy was not effective for the fully deglycosylated (QQQQ) mutant (Fig. 6).

Structure and TNF Binding Activity of TNFR-IgG1 Glycosylation Mutants

Although the signal sequence exchange strategy described above provided a method that allowed for the secretion of TNFR-IgG1 glycosylation site mutants, we speculated whether the secreted proteins were properly folded. To answer this question, stable CHO cell lines, expressing several of the TNFR-IgG1 glycosylation mutants, were constructed in order to produce sufficient quantities for ligand binding studies. Recombinant proteins representing the NNQQ, NSNQ, and QSNQ mutants were purified from growth-conditioned cell culture medium using protein A affinity chromatography. As expected (Fig. 7), the molecular mass of the glycosylation variants correlated with the number of intact glycosylation sites. Thus, the variants with two glycosylation sites deleted (NNQQ and NSNQ) were smaller in molecular weight than the wild-type (NNNN) proteins and larger in molecular mass than the QSNQ variant that possessed only one glycosylation site.

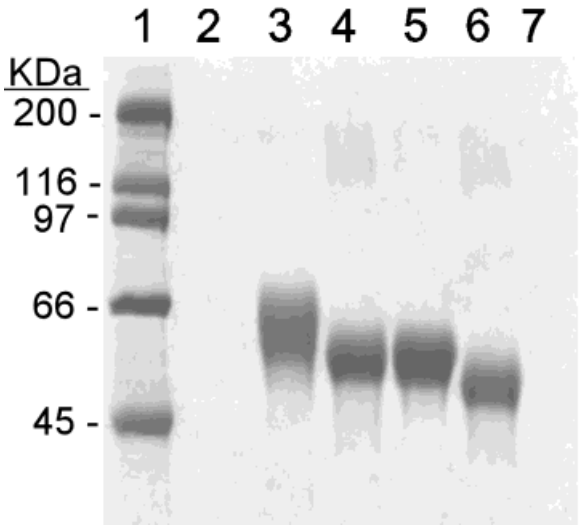

Fig. 7. Characterization of TNFR-IgG1 glycosylation mutants purified for receptor binding studies. TNFR-IgG1 glycosylation site mutants were purified by protein A affinity chromatography from growth conditioned cell culture supernatants of stable transfected CHO cell lines. The purified proteins were treated with SDS-PAGE sample buffer containing beta-mercaptoethanol (**lanes 3–6**) and resolved by SDS-PAGE. The resulting gel was stained with Coomassie blue. Each lane was loaded with 3μg of purified protein. Lane 3 contained fully glycosylated TNFR-IgG1; lane 4 contained the NNQQ glycosylation site mutant; lane 5 contained the NSNQ glycosylation site mutant; and lane 6 contained the QSNQ glycosylation site mutant. The mobilities of molecular weight standards are shown in **lane 1**; **lanes 2** and **7** contained sample buffer alone.

TNF binding studies were carried out to determine whether the secreted glycosylation variants were folded into a functionally relevant conformation. For this purpose, the three glycosylation mutants illustrated in Figure 7 were evaluated in a binding assay (Fig. 8) using (^{125}I)-labeled TNF by a method similar to that described by Ashkenazi et al [1991]. All three mutants bound the (^{125}I)-labeled TNF with EC_{50} values (Table II) comparable to that observed for fully glycosylated TNFR-IgG1. These studies suggested that neither glycosylation sites nor the tPA signal/pro sequence affected the interaction of TNFR-IgG1 with ligand.

DISCUSSION

Previous studies [Brunner et al., 1992; Collier et al., 1993; Dash et al., 1994; Gallagher et al., 1988; Kobayashi et al., 1994; Leavitt et al., 1977; Machamer and Rose, 1988] have demonstrated that deletion of N-linked glycosylation sites often impairs the intracellular transport and secretion. In the present study, we similarly found that deletion of glycosylation sites from TNFR-IgG1 impaired glycoprotein export, but discovered that the defect in secretion could, in large part, be overcome by replacement of the TNFR signal sequence with the signal/pro sequence of human tPA. To our knowledge, this is the first report of an effective strategy to rescue the secretion of glycosylation site mutants from mammalian cells. Remarkably, the complex events that occur during folding and dimerization were preserved in the glycosylation site mutants as well as ligand binding ability. Previous studies [Fiedler and Simons, 1995; Gething and Sambrook, 1992; Haas and Wabl, 1983; Helenius, 1994; Roth and Pierce, 1987; Rothman, 1989] have suggested glycosylation mutants were retained in the ER because they were insoluble, misfolded, or sequestered by chaperones or foldases performing quality control functions. Because TNFR-IgG1 is an artificially created chimeric protein, it seems likely that it might possess unusual structural features that promote interactions with chaperones and foldases, resulting in sequestration in the ER. While sequestration by chaperones and foldases may be the mechanism of ER retention

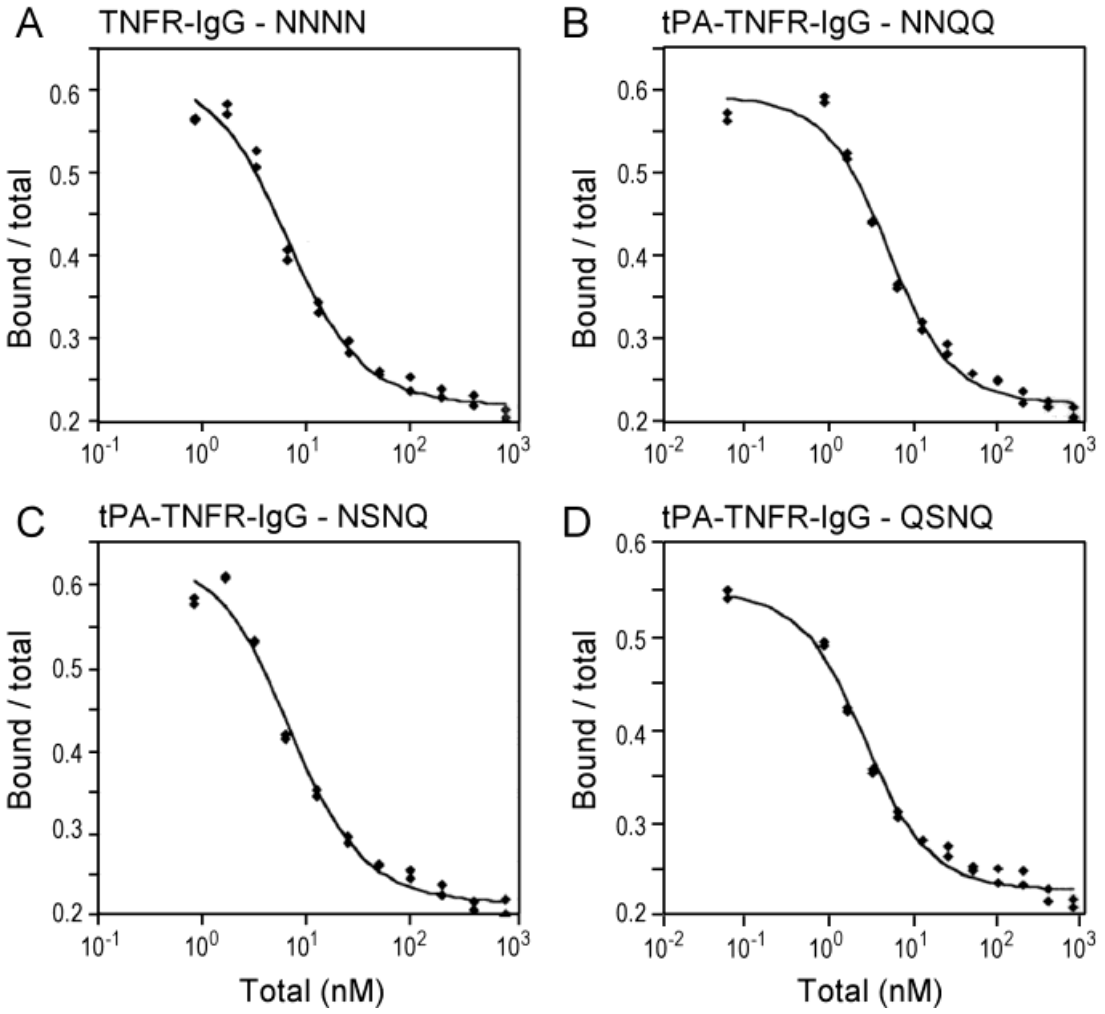

Fig. 8. Competitive binding of (^{125}I)-labeled TNF to TNFR-IgG1 glycosylation mutants. Purified TNFR-IgG1 and TNFR-IgG1 glycosylation site mutants were captured onto microtiter plates coated with affinity purified goat antibodies to human IgG Fc domain. The captured receptor chimeras were reacted with (^{125}I)-labeled TNF along with varying concentrations of unlabeled TNF. **A:** Binding of TNF to fully glycosylated TNFR-IgG1. **B:** Binding to the NNQQ glycosylation mutant. **C:** Binding to the NSNQ glycosylation mutant. **D:** Binding to the QSNQ glycosylation mutant.

of TNFR-IgG1 glycosylation mutants, it is not clear how the replacement of N-terminal sequences can overcome these interactions.

Previous studies (Brousseau and Etcheverry, Genentech, Inc., personal communication) suggested that the tPA signal/pro sequence was somewhat unique in comparison with other signal sequences (HSV-1 gD,hGH) that failed to improve the secretion efficiency of TNFR-IgG1. The mechanism by which the tPA signal/pro sequence overcomes ER retention may relate to its complex structure. The leader sequence of tPA is unusual in that it contains a 23 amino acid signal sequence and a 12 amino acid prosequence containing a 3 amino acid exopeptidase cleavage site (Fig. 1B). Previous studies [Berg and Grinnell, 1991] have shown that the signal sequence is cleaved co-translationally in the endoplasmic reticulum and that the prosequence is removed in the GA by cleavage at a furin processing site [Barr, 1991; Steiner et al., 1992]. The fact that the endo H sensitive, intracellular forms of the TNFR-IgG1 glycosylation mutants expressed with the tPA signal/pro sequence possessed higher molecular weight than the secreted products, demonstrates that removal of the pro-sequence from glycosylation mutants occurs during export through the GA. Based on these observations, an intriguing possibility is that the tPA pro-sequence represents

a structural element able to promote ER to GA transport of the TNFR-IgG1 glycosylation mutants.

Several alternate mechanisms might account for the activity of the tPA signal/pro sequence. First, the tPA signal/pro sequence may have a direct effect on protein folding or solubility that overcomes the protein structure alteration caused by deletion of glycosylation sites. A second possibility is that replacement of the wild-type TNFR signal sequence with the tPA signal/pro sequence results in elimination of a chaperone (e.g., calnexin) binding site at the amino terminus such as that reported [Li et al., 1996] for the expression of HIV-1 gp120. A third possibility is that the tPA signal/pro sequence contains a recognition signal that causes the nascent proteins to efficiently partition into vesicles that mediate ER to GA transport. Additional mutagenesis studies are in progress to define the structural elements (e.g., the furin processing site) that enable the tPA signal/pro sequence to facilitate transport out of the ER (Brousseau and Etcheverry, Genentech, Inc., personal communication).

Although it was possible to achieve secretion of TNFR-IgG1 variants with up to three of the four glycosylation sites deleted (NNQQ, NSNQ, and QSNQ), an important question to be answered was whether changing the asparagine at one particular glycosylation site could affect the occupancy as well as carbohydrate structure on one or more of the remaining glycosylation sites. To answer this question, peptide mapping and carbohydrate sequencing studies of the mutants were carried out to compare their N-linked glycosylation site occupancy with wild-type TNFR-IgG1 (Peers and Keck, unpublished results). These peptide maps demonstrated that use of the remaining N-linked sites was not affected by the removal of the glycosylation sites. Mass spectroscopy (MALDI-TOF) analysis of mutant NNQQ (data not shown) confirmed that the occupied sites have the same complex carbohydrate structure as TNFR-IgG1 (Peers and Keck, unpublished results).

Another question that deserves consideration is whether or not the secreted glycosylation variants were properly folded. And, did incorporation of the tPA signal/pro sequence allow these variants to bypass quality control systems and export misfolded and inactive proteins? This question was answered in structural studies and ligand binding studies where it was found that all of the glycosylation variants tested formed covalently bound homodimers and bound TNF with relative binding affinities similar to fully glycosylated TNFR-IgG1. This study raised the possibility that the QSNQ mutant might bind TNF with a slightly higher affinity (EC_{50} = 2.34 nM ± 0.22) than wild-type TNFR-IgG1 (EC_{50} = 6.66 nM ± 0.73); however, more detailed studies are required to confirm this observation. If ER retention is attributable to the chaperonen/foldase quality control system, this study demonstrates that abnormal transport and secretion in bioengineered molecules do not necessarily correlate with abnormal function.

The observation that TNFR-IgG1 glycosylation mutants retain much of their ligand binding ability is consistent with structural studies [Banner et al., 1993] showing that the glycosylation sites on TNFR are far removed from the TNF binding site. However, our inability to produce a fully deglycosylated variant of TNFR-IgG1 that was secreted from the cells is puzzling. At this point, we are not certain whether the failure to secrete this mutant is because the tPA signal/pro sequence was not able to overcome all ER retention of all glycosylation mutants, or whether the fully deglycosylated protein is truly misfolded (e.g., mismatched disulfide bonds) and perhaps retained at a different step in the folding pathway.

The present analysis of the biosynthesis of TNFR-IgG1, as well as previous studies characterizing the biosynthesis and secretion of HSV-1 gD and HIV-1 gp120 [Berman, 1985; Berman et al., 1989; Lasky et al., 1986], suggest that the intracellular transport of bioengineered variants of membrane glycoproteins proteins are often less efficient than that authentic membrane or secreted proteins. In the three cases we have studied–HSV-1 gD, HIV-1 gp120, and TNFR-IgG1—the truncated proteins accumulate in the ER, and the rate limiting step in secretion is transport from the ER to the GA. Recent studies [Hilton et al., 1995] have suggested that ER retention may be a naturally occurring phenomenon that, in some cases (e.g., the erythropoeitin receptor), provides a mechanism that can be utilized by cells to regulate the expression of cell surface receptors. Our results suggest ER to GA transport is a highly constrained phenomenon and suggest that mul-

tiple strategies may have evolved to regulate secretion efficiency. One strategy appears to relate to modulation of the association between nascent proteins and chaperones and foldases (e.g., BIP and grp94), which can sequester nascent proteins in the ER. Another mechanism highlighted in this study may be acquisition of sequences such as the tPA signal/pro sequence that can somehow bypass the ER retention mechanism. Understanding the mechanism of ER retention may have major implications in the development of bioengineered molecules of pharmacologic potential. Further studies are in progress to determine whether the glycosylation site mutants described in this paper differ in their pharmacologic half-life and bioavailability in vivo.

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