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Functional analysis of the posttranslational modifications of the death receptor 6

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ABSTRACT

Death receptor 6 (DR6/TNFRSF21) is a death domain-containing receptor of the TNFR superfamily with an apparent regulatory function in hematopoietic and neuronal cells. In this study we document that DR6 is an extensively posttranslationally modified transmembrane protein and that *N*- and *O*-glycosylations of amino acids in its extracellular part are mainly responsible for its approximately 40 kDa mobility shift in SDS polyacrylamide gels. Site-directed mutagenesis confirmed that all six extracellular asparagines are *N*-glycosylated and that the Ser/Thr/Pro cluster in the "stalk" domain juxtaposed to the cysteine-rich domains (CRDs) is a major site for the likely mucine-type of *O*-glycosylation. Deletion of the entire linker region between CRDs and the transmembrane domain, spanning over 130 amino acids, severely compromises the plasma membrane localization of DR6 and leads to its intracellular retention. Biosynthetic labeling with radiolabeled palmitate and side-directed mutagenesis also revealed that the membrane-proximal Cys368 in the intracellular part of DR6 is, similarly as cysteines in Fas/CD95 or DR4 ICPs, *S*-palmitoylated. However, palmitoylation of Cys368 is apparently not required for DR6 targeting into Brij-98 insoluble lipid rafts. In contrast, we show that *N*-glycosylation of the extracellular part might participate in directing DR6 into these membrane microdomains.

1. Introduction

Death receptor-6 was till recently one of the few "orphan" receptors of the TNFR superfamily. TNFR receptors are recognized by the presence of 1–4 cysteine-rich domains in their extracellular parts. Together with their ligands, predominantly expressed by hematopoietic cells, they participate in regulating the proliferation, survival or apoptosis of various, not exclusively hematopoietic, cells [1]. DR6 was identified as a new member of the death receptor subfamily, which is distinguished by the presence of an α-helical structural motif called the death domain in the intracellular part [2]. Some of the death receptors, such as Fas/CD95 or the TRAIL receptors TRAIL-R1/DR4 and TRAIL-R2/DR5, are potent inducers of apoptosis, while others such as DR3 or EDAR participate in the regulation of T cell activation or play a role during development [3,4].

Cysteine-rich domains of DR6 as well as its death domain and the C-terminal α-helical CARD-like region are highly conserved in vertebrates from zebrafish to humans. Overexpression of DR6 in some cell lines leads to apoptosis and/or to the activation of NFκB and stress kinases of the JNK/SAPK family [2,5,6].

The unavailability of the DR6 ligand has restricted studies examining DR6 function mainly to analyses of DR6 knockout mice. DR6 is not, similarly as other death receptors, required for the development or survival of mouse embryos, but its genetic inactivation enhanced the proliferation of CD4+ T cells and the production of Th2 cytokines [7,8]. Similarly, DR6-deficient B lymphocytes were hyperproliferative following various stimuli and showed attenuated stimulation-induced cell death [9]. The enhanced activation of DR6-deficient T and B cells was also reflected in more severe graft-versus-host disease induced by allogenic bone marrow transplantation into irradiated recipient mice [10]. The development and progression of autoimmune or allergic responses, such as experimental autoimmune encephalomyelitis or allergic airway inflammation, is attenuated in DR6 knockout mice [11,12]. However, recently the cleaved off extracellular part of APP or APLP2 proteins was reported to function as a DR6 ligand and to trigger DR6-dependent death of neurons and/or pruning of their axons [13].

DR6 expression is also markedly increased in several human tumor-derived cell lines. Increased DR6 expression in PC3 or DU145 prostate carcinoma cells is accompanied by enhanced NFκB signaling and increased $BclX_L$ expression [14]. In another prostate carcinoma cell line, LnCAP, DR6 expression was induced in a TNFα/NFκB-dependent manner. Increased DR6 expression on tumor cells could have functional consequences as matrix metalloproteinase-14 is able to cleave DR6 from the cell surface, and the shed extracellular part of DR6 was suggested to attenuate the *in vitro* differentiation of monocytes into dendritic cells, which can contribute to tumor evasion from the immune system [15].

In this communication we document that DR6 is *S*-palmitoylated on its membrane-proximal cysteine and its extracellular part is extensively *N*- and *O*-glycosylated. We show that these DR6 modifications are

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related to each other and might affect the plasma membrane localization of DR6.

2. Materials and methods

2.1. Plasmids and antibodies

The coding region of human DR6 was amplified by RT-PCR from total HeLa RNA, prepared by TRIZOL Reagent (Invitrogen) according to the manufacturer's instructions. The PCR product was cloned into pBluescript SK vector (Stratagene) and sequenced. For bacterial expression, the DR6 intracellular fragment corresponding to amino acids 371–655 was cloned into the pET-15b expression vector (Novagen), generating a construct with an N-terminal histidine tag. For expression in eukaryotic cells, DR6 cDNA was subcloned into the pcDNA3 vector (Invitrogen).

Site-directed mutagenesis was used to replace the asparagine residues of human DR6 at positions 82, 141, 252, 257, 287 and 289 with glutamines, the cysteine residue at position 368 with valine, and for internal deletions of the DR6 linker region (amino acids 212–349). Mutagenesis was performed with the QuickChange Site-Directed Mutagenesis Kit (Stratagene) with the primers summarized in Suppl. Table 2. Multiple point mutants were prepared by consecutive site-directed mutagenesis.

Mouse monoclonal antibodies against the extracellular part of DR6 were prepared using the standard techniques from splenocytes of mice immunized with the chimeric fusion protein of the extracellular part of DR6 (amino acids 42–335) and the Fc portion of human immunoglobulin IgG1 (Alexis). To raise mouse monoclonal or rabbit polyclonal antibodies directed against the intracellular domain of DR6, a purified His-tagged fragment of human DR6 (amino acids 370–655) expressed in E. coli was used as an immunogen. For some experiments, rabbit polyclonal antibody directed against the N-terminal peptide of mature DR6 (amino acids 42–56) was used as a control (BD PharMingen). Rabbit polyclonal anti-CD59 antibody (MEM-43) was kindly provided by Prof. V. Horejsi.

2.2. Cell culture, transfections and down-regulation of DR6 expression

The human T cell lines Jurkat and HuT78, the B cell lines KM3 and IM-9, the myeloid cell lines THP-1 and HL60 and the prostate carcinoma cell line LnCAP were maintained in RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum. The human embryonic kidney cell lines HEK293 and HEK293FT, human immortalized keratinocytes NCTC and the cervical-carcinoma cell line HeLa were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS. NCTC cells were provided by Dr. L. LoMuzio; all other cell lines were obtained from either ATCC or from the cell line collection of the Institute of Molecular Genetics.

Transfections of HeLa or HEK293 cells were performed using LF20020 (Invitrogen) or Fugene HD (Roche), as described by the manufacturer. Two independent DR6 siRNA oligonucleotides (Ambion) or control luciferase siRNA (Dharmacon) were used for lipofectamine RNAiMAX-mediated down-regulation of DR6 expression in NCTC cells (Suppl. Table 1). TNFα used for the treatment of LnCAP cells was purchased from R&D Systems.

2.3. Immunoprecipitation

The cells were harvested, washed with phosphate-buffered saline and lysed in ice-cold lysis buffer (1% NP-40, 20 mM Tris Cl pH 7.5, 100 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM Na_3VO_4, 10% glycerol), supplemented with protease inhibitors (Complete® protease inhibitor cocktail, Roche). After solubilizing for 30 min on ice, the lysate was pre-cleared by centrifugation at 16,000×*g* for 30 min. The resulting supernatant was incubated either with anti-DR6 mAb covalently coupled to CNBr-activated Sepharose beads (GE Healthcare) or with rabbit anti-DR6 antibodies bound to protein A/G Sepharose (Pierce) for 1 h at 4 °C. After three washes with 10 volumes of the lysis buffer, the immunoprecipitates were directly eluted with Laemmli sample buffer and analyzed by Western blotting using either rabbit or mouse anti-DR6 antibodies.

2.4. Confocal microscopy and flow cytometry

HeLa cells transfected with DR6 mutants and grown on cover slips were permeabilized in a −20 °C methanol bath for 10 min and then for 5 s in cold acetone. After washing in PBS the slides were incubated with anti-DR6 mAb for 30 min, followed by 30 min incubation with 4 μg/ml Alexa488-conjugated goat anti-mouse IgG (Molecular Probes). The samples were mounted in moviol with DRAQ5® (Axxora) and viewed with a Laserscan confocal microscope (Leica TCS SP).

The cells for flow cytometry analysis were harvested and incubated on ice with a blocking solution (PBS + 0.2% gelatine and 0.1% sodium azide=PBS-GA) containing 20% heat-inactivated human serum for 10 min. After blocking, the samples were incubated on ice in PBS-GA with anti-DR6 mAb (5 μg/ml) for 30 min, followed by a 30-min incubation on ice in PBS-GA with 4 μg/ml phycoerythrin-conjugated goat anti-mouse IgG1 (SouthernBiotech). After final washing, the surface expression of DR6 on living cells (Hoechst33258 low) was analyzed by flow cytometry on a LSRII (BD Biosciences).

2.5. Cell treatments and endoglycosidase digestions

The cells were cultured overnight in the presence of the N-glycosylation inhibitor tunicamycin (final concentration 5 μg/ml, Sigma), or in the presence of 2 mM benzyl-2-acetamido-2-deoxy-α-D-galactose (benzyl-*O*-GalNAc, Sigma), an inhibitor of the formation of *N*-acetylgalactosamine-*O*-Ser/Thr-linked glycoconjugates, or alternatively in the presence of 200 μM 2-bromopalmitate (Sigma), a palmitate analogue and an inhibitor of *S*-palmitoylation.

To perform endoglycosidase digestions, the immunoprecipitated DR6 was denatured and eluted from the anti-DR6-Sepharose beads by incubation in 1× Glycoprotein Denaturing Buffer (New England Biolabs) at 95 °C for 10 min. *N*-linked glycans were cleaved off with PNGase F (*N*-glycosidase F, NEB) and *O*-linked oligosaccharides with endo-α-*N*-acetylgalactosaminidase (*O*-glycosidase, NEB) and neuraminidase (NEB) by 20 h incubation at 37 °C in 1× G7 Reaction Buffer (NEB) supplemented with 1% NP40.

2.6. Biosynthetic labeling with [3H]palmitate

HEK293FT cells (2×10^7 cfu) were transfected with DR6 expression plasmids, starved at 37 °C in 50 ml of plain RPMI1640 medium for 1 h and then 0.5 mCi of radiolabeled palmitate ([9,10(n)-3H]palmitic acid, specific activity 30–60 Ci/mmol; PerkinElmer Life and Analytical Sciences) was added. After 3 h at 37 °C the cells were washed and solubilized in a 1% NP40 lysis buffer, and the postnuclear supernatant was used for immunoprecipitation followed by SDS-PAGE. The wet gel was treated with Amplify solution (GE Healthcare) according to the manufacturer's recommendations, dried, and subjected to fluorography.

2.7. Density gradient ultracentrifugation

HEK293 cells (2×10^7 cfu) transfected with appropriate DR6 (wt or mt) expression plasmids or NCTC cells (10^8 cfu) were harvested, washed with PBS and lysed in 0.5 ml of ice-cold lysis buffer (1% Brij98, 20 mM Tris Cl pH 8.2, 100 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM Na_3VO_4), supplemented with protease inhibitors (Complete® protease inhibitor cocktail, Roche), on ice for 30 min. 0.5 ml of the lysate was then mixed at a 1:1 ratio with ice-cold 80% (wt/vol) sucrose diluted in lysis buffer and transferred into 5-ml ultracentrifugation

tubes, overlaid with 3.5 ml of ice-cold 30% sucrose diluted in lysis buffer and finally with 0.5 ml of sucrose-free lysis buffer. After centrifugation at 50,000 rpm in a MLS-50 rotor (cca 268,000 ×*g*; Beckman Coulter) for 18 h at 4 °C, 0.7 ml fractions were collected from the top to the bottom of each tube and analyzed by Western blotting.

3. Results

3.1. DR6 is expressed in two forms of apparent molecular masses 90 kDa and 110 kDa

Although from mouse knockout data the death receptor-6 is thought to have a dampening function in regulating the immune response, it was reported to be also expressed in some human, mainly prostate-derived, tumor cell lines [14]. In our screening for additional cells that might express DR6, we reproducibly observed in the cell lysates of all analyzed cell lines, of both hematopoietic and non-hematopoietic origin, a 68–70 kDa band (termed p70) that would correspond to the predicted molecular mass of human DR6 (68 kDa or 72 kDa including the putative signal peptide). This band was stained with polyclonal antibodies that map both to the N-terminal peptide (amino acids 42–56, e.g. Abcam ab8417) and to the cytoplasmic part of DR6 (amino acids 371–655, e.g. Abcam ab47180 or our own rabbit polyclonal antibody, marked by asterisks in Fig. 1A). However, wild-type DR6 exogenously expressed in HEK293 cells predominantly yielded higher molecular weight bands of apparent molecular masses 85–90 kDa (termed p90) and 105–110 kDa (termed p110), but not the p70 band (not shown). In addition, using our own monoclonal antibodies raised against the extracellular part of DR6, we were able to immunoprecipitate from some cell lines (KM3, Jurkat, NCTC) the 110 kDa and, to a lesser extent, the 90 kDa forms of DR6 (marked by arrows in Fig. 1A) but almost none of the 70 kDa protein. In order to resolve this uncertainty regarding the expression and apparent molecular weight of DR6, we also determined the cell surface expression of DR6 in the tested cell lines by flow cytometry (Fig. 1B) and quantified the relative amount of *DR6* mRNA by real-time qRT-PCR (Suppl. Fig. 1); these results closely matched those from the immunoprecipitation of the DR6 110/90 kDa forms, suggesting that the p70 protein is not the major functional form of DR6 and its real identity is uncertain (at least in cells that we used in this study).

These findings are supported by two other independent lines of experimental evidence. First, siRNA-mediated knockdown of *DR6* mRNA in NCTC cells led to the disappearance of only the 110/90 kDa forms and not the 70 kDa band (Fig. 1C). Second, according to the published observation that TNFα induces the expression of DR6 through the activation of NF-κB [14], the p90 and p110 DR6 forms were strongly upregulated by the cultivation of LnCAP prostate carcinoma cells in the presence of TNFα, whereas the expression of the p70 protein again remained unaffected (Fig. 1D). Summing these data together, it appears that not the calculated 70 kDa form but the 110 kDa and, to a lesser extent, the 90 kDa bands are the major if not the only naturally expressed forms of DR6.

3.2. DR6 is a palmitoylated protein with an extensively glycosylated extracellular part

The significant difference between the predicted (70 kDa) and actual (110 kDa and 90 kDa) molecular masses of DR6 suggests the presence of possible posttranslational modification(s) that modulates its relative mobility in SDS-PAGE. These modifications most probably reside in the extracellular region of DR6 as the deletion mutant lacking most of the DR6 intracellular part modulated its relative mobility in SDS-PAGE in a similar way as did wild-type DR6 (not shown). The TNFR superfamily members are often subjected to glycosylation, and therefore we searched the extracellular sequence of DR6 for potential *N*- and mucin-type *O*-glycosylation sites using NetNGlyc 1.0 and NetOGlyc 3.1 predictions, respectively [16]. Human DR6 contains six potential *N*-linked oligosaccharide chain sites (Asn82, Asn141, Asn252, Asn257, Asn278 and Asn289) and multiple potential *O*-linked oligosaccharide chain sites with a greater preference for Thr213, Thr221, Thr227, Thr238, Thr245 and Thr254 (Suppl. Fig. 2A).

For assessing the relative contribution of *N*- and *O*-glycosylation, we combined cell treatment with known inhibitors of *N*- and *O*-glycosylation, tunicamycin and benzyl-2-acetamido-2-deoxy-α-D-galactose (benzyl-*O*-GalNAc) respectively, with the in vitro deglycosylation of the immunoprecipitated DR6. For *in vitro* enzymatic deglycosylation, we used either PNGase F (*N*-glycosidase F), which removes *N*-linked oligosaccharides by cleaving between the innermost GlcNAc and Asn residues, or a mixture of neuraminidase and endo-α-*N*-acetylgalactosaminidase (*O*-glycosidase), which removes the terminal sialic acid residues or desialyated core 1 and core 3 *O*-linked disaccharides attached to Ser/Thr residues, respectively.

Cell treatment with glycosylation inhibitors as well as enzymatic deglycosylation, either alone or in various combinations, led to a distinct but not complete reduction of the apparent molecular mass of DR6 (Fig. 2A). PNGase F and tunicamycin interventions significantly enhanced the mobility of both p110 and p90, indicating that these forms of DR6 contain *N*-linked oligosaccharides (Fig. 2A, lanes 5 and 13). In contrast, the *O*-glycosylation inhibitor benzyl-*O*-GalNAc and *in vitro* *O*-linked deglycosylation of the immunoprecipitated DR6 partly enhanced the mobility of only the p110 form, which argues for the presence of *O*-linked oligosaccharides only in the p110, presumably a more mature form of DR6 (Fig. 2A, lanes 4 and 7). Subsequent usage of both of these de-*O*-glycosylation interventions shifted mobility of the p110 form even further implicating non-overlapping modes of the action of these reagents (Fig. 2A, lane 8). Both enzymatic *N*-linked deglycosylation and tunicamycin treatment shifted the mobility of the p90 band to approximately 70 kDa, suggesting that p90 might be an *N*-only-glycosylated intermediate form of DR6 (Fig. 2A, lanes 5 and 13). The combination of tunicamycin, benzyl-*O*-GalNAc and *O*-glycolytic enzymes enhanced DR6 gel migration most efficiently (Fig. 2A, lanes 18 and 20). Thus, it appears that the p110 form of DR6 is both *N*- and *O*-glycosylated and that these glycosylations largely account for the apparent approximate 40 kDa shift in its calculated mobility.

Glycosylation prediction analysis points to six potentially *N*-glycosylated asparagines in the DR6 extracellular part and a cluster of *O*-glycosylated serines and/or threonines between amino acids 212 and 254 of the DR6 precursor (Suppl. Fig. 2A). Individual and consecutive mutagenesis of all asparagines (starting from Asn82) to glutamines in the DR6 extracellular part revealed that each of them is *N*-glycosylated (gradual shift in the apparent molecular weight), and the migration of the ultimate DR6 mutant reflected the DR6 mobility in tunicamycin-treated cells (Fig. 2B). These data lead us to conclude that all six asparagines in the extracellular part of DR6 are *N*-glycosylated.

As Fas/CD95 and TRAIL-R1/DR4 were recently described as palmitoylated proteins targeted to lipid rafts [17,18], we asked whether this membrane domain targeting- and trafficking-related posttranslational modification can also occur in DR6. Human and mouse DR6 proteins contain a conserved cysteine residue in the membrane-proximal intracellular region (Cys368 in the human DR6 precursor), which could be potentially *S*-palmitoylated (Suppl. Fig. 2C). HEK293FT cells were transfected with DR6 and DR6 (C368V) mutants, cultured in medium containing radioactive [3H] palmitate, lysed and immunoprecipitated with DR6 antibody. Autoradiography of the SDS-PAGE-separated immunoprecipitates revealed that [3H]palmitic acid is incorporated into DR6 and slightly more efficiently also into DR6(6xNQ) mutant but not into the DR6(C368V) mutant, indicating that Cys368 is indeed being palmitoylated (Fig. 2C, lanes 1–4). Specificity of DR6 palmitoylation was further confirmed using a competitive palmitoylation inhibitor 2-bromopalmitate (Fig. 2C, lanes 6, 7).

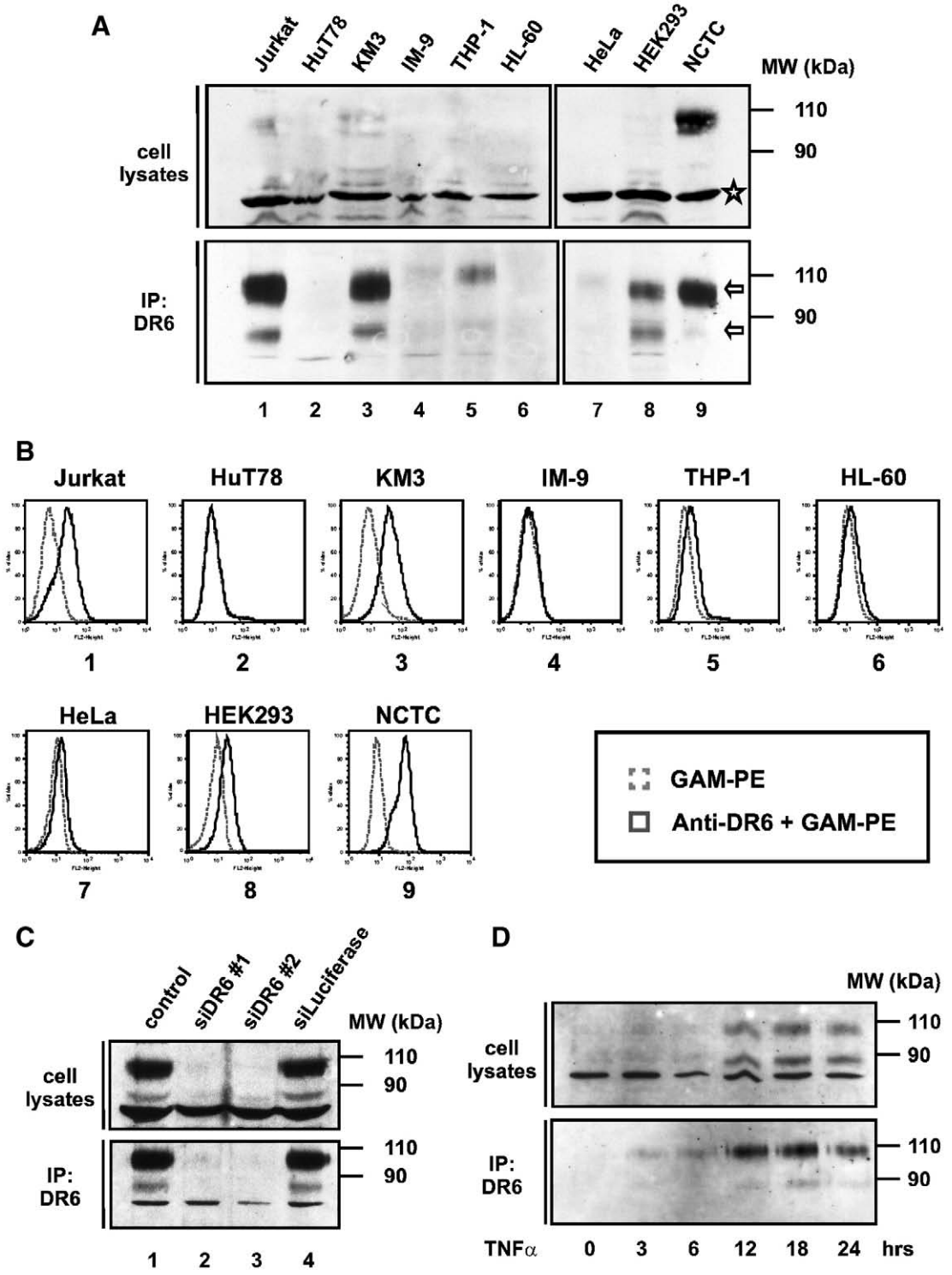


Fig. 1. Death receptor 6 is expressed at the cell surface of various cell lines as a 110 kDa protein. A. The indicated cell lines (approximately 3×10^7 cfu) were lysed and DR6 was either immunoprecipitated from the lysate with anti-DR6(ECP) mAb (lower panel) prior to Western blotting or the cell lysate was directly analyzed by Western blotting using rabbit anti-DR6(ICP) antibodies (upper panel). B. Cell surface expression of DR6 on these cell lines was analyzed by flow cytometry using anti-DR6(ECP) mAb. C. NCTC cells were transfected with control luciferase or two different DR6 siRNAs, and DR6 from their cell lysates was immunoprecipitated with anti-DR6(ECP) mAb and/or analyzed by anti-DR6(ICP) Western blotting. D. LnCAP prostate carcinoma cells were treated with TNFα (20 ng/ml) for the indicated time periods then lysed, and DR6 was immunoprecipitated with anti-DR6(ECP) mAb and revealed by Western blotting.

3.3. The extensively O-glycosylated linker region between DR6 CRDs and the transmembrane part is required for the plasma membrane localization of DR6

Having established the presence of multiple posttranslational modifications in DR6, we were eager to uncover their function. In addition to the already prepared mutants in the *N*-glycosylation and palmitoylation sites, we aimed to target the *O*-glycosylation sites as well. In contrast to most of its TNFR kin, DR6 harbors an unusually long (approximately 130 amino acids) linker region between CRDs and the transmembrane domain, which also contains the majority of the predicted mucin-type *O*-linked oligosaccharide chain sites, mainly

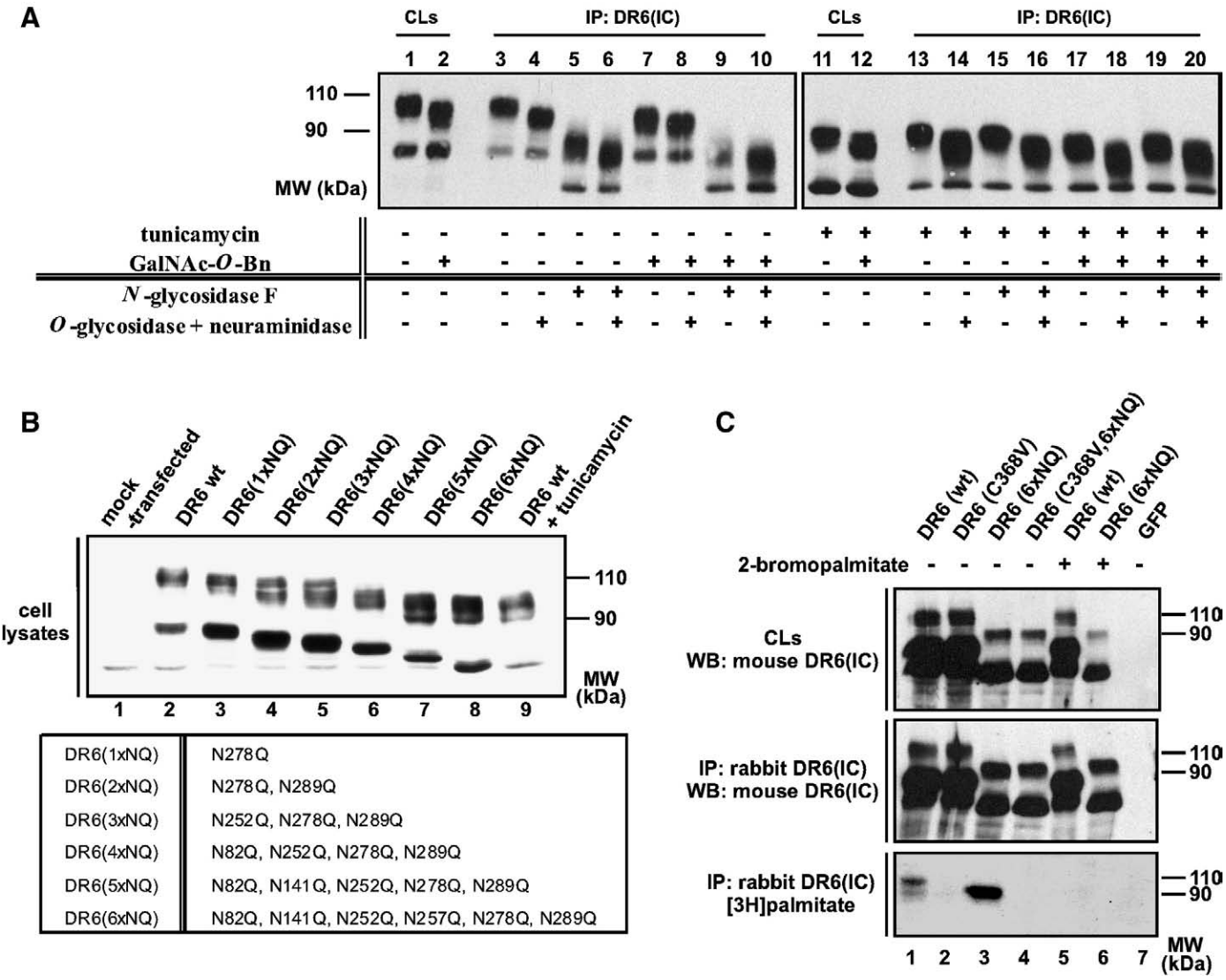
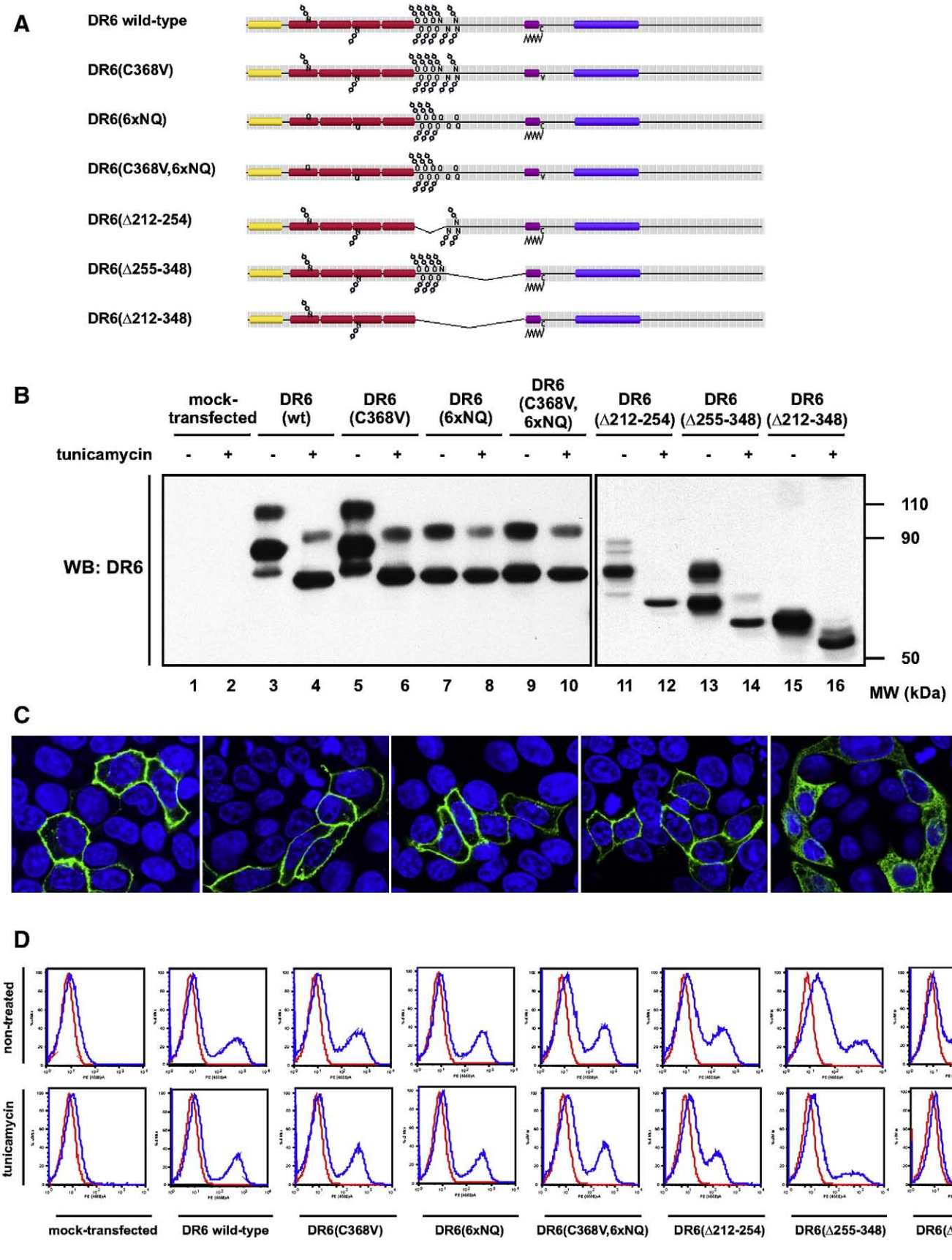


Fig. 2. DR6 is an extensively N- and O-glycosylated and S-palmitoylated protein. A. DR6-transfected HEK293FT cells were cultured with 5 μg/ml of tunicamycin and/or 2 mM benzyl-*O*-GalNAc for 15 h, lysed and then DR6 immunoprecipitated with mouse monoclonal anti-DR6(IC) antibody was, where indicated, subjected to glycolytic treatment and analyzed by Western blotting using rabbit anti-DR6(EC) polyclonal antibodies (Pharmingen). B. HEK293FT cells were transfected with plasmids encoding consequent DR6 (N→Q) mutants, lysed and analyzed by Western blotting with anti-DR6 rabbit pAbs. The mutated Asn in individual DR6 mutants are displayed in the inset. C. HEK293FT cells were transfected with the indicated DR6 expression plasmids and biosynthetically labeled with [3H]palmitate, then the DR6 in the cell lysates (upper panel) or the rabbit anti-DR6(IC) immunoprecipitates (middle and lower panels) were analyzed by SDS-PAGE followed by Western blotting with anti-DR6 antibodies and fluorography.

clustered between amino acids Thr212 and Thr254 (see Suppl. Fig. 2B). Similarly clustered Ser-/Thr-/Pro-rich sequences, called "stalk regions", were found in some other TNFR receptors (as TNFR2, CD30 or NGFR) and are also predicted to be extensively *O*-glycosylated (Suppl. Table 1). As multiple *O*-glycosylation sites are predicted with a higher probability mainly in the stalk region, we targeted it as well as its complementary part and the entire linker region for deletion. The complete set of DR6 mutants (Fig. 3A) was used for analyzing their effect on DR6 plasma membrane localization. The expression and proper folding of the DR6 variants transfected into HEK293FT cells were confirmed by immunoprecipitation with conformation-specific monoclonal antibodies to DR6 and by Western blotting. All proteins were expressed and, when applicable as revealed by tunicamycin treatment, also *N*-glycosylated. Mobility in SDS-PAGE and the membrane localization of the *S*-palmitoylation-deficient mutant DR6 (C368V) were not different from wild-type DR6, and the fully *N*-glycosylation-deficient mutant DR6(6xNQ) was also localized at the plasma membrane (Fig. 3). Deletion of the stalk region (amino acids 212–254) led to a large reduction in the amount of fully modified DR6 corresponding to the p110 form in wild-type DR6 and to the predicted shift in the mobility of the *N*-only-glycosylated form corresponding to the p90 form in wild-type DR6 (Fig. 3B, lanes 11–12). In contrast, removing the complementing sequence in the linker domain (amino acids 255–348) did not affect additional posttranslational modifications of DR6, arguing for the stalk domain as the major likely *O*-glycosylated region in the extracellular part of DR6 (Fig. 3B, lane 11). Notably, the efficient DR6/Δ255–348 presentation at the plasma membrane (Fig. 3D, compare the histograms of wild-type DR6 and DR6/Δ255–348) can be related to its enhanced *O*-glycosylation. As predicted, DR6/Δ212–348 contains just two *N*-glycosylation sites, and upon tunicamycin treatment it migrates in agreement with the calculated molecular weight of approximately 55 kDa (Fig. 3B, lanes 15–16). However, in contrast to the partial deletion mutants, removing the entire linker region from the DR6 extracellular part significantly affected the behavior of this DR6 mutant. It was drastically less mobilized to the plasma membrane than wild-type DR6 or the partial deletion linker region mutants and remained stacked most probably in the Golgi system (Fig. 3C, D). Thus, the linker region (or its major part) is required for the proper transport of DR6 to the plasma membrane.


A
DR6 wild-type
DR6(C368V)
DR6(6xNQ)
DR6(C368V,6xNQ)
DR6(Δ212-254)
DR6(Δ255-348)
DR6(Δ212-348)
B
mock-transfected
DR6 (wt)
DR6 (C368V)
DR6 (6xNQ)
DR6 (C368V, 6xNQ)
DR6 (Δ212-254)
DR6 (Δ255-348)
DR6 (Δ212-348)
tunicamycin
WB: DR6
110
90
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
MW (kDa)
C
D
non-treated
tunicamycin
mock-transfected
DR6 wild-type
DR6(C368V)
DR6(6xNQ)
DR6(C368V,6xNQ)
DR6(Δ212-254)
DR6(Δ255-348)
DR6(Δ212-348)

3.4. N-glycosylation but not S-palmitoylation of DR6 might affect its targeting to lipid rafts

The palmitoylation of Cys368 juxtaposed to the transmembrane region and the targeting of palmitoylated Fas/CD95 and TRAIL-R1 to lipid rafts raised a question about the plasma membrane sublocalization of DR6. To analyze in which membrane compartment endogenous DR6 resides, human keratinocyte-derived cells NCTC (the cell line with the highest DR6 expression that we detected) were lysed in Brij98 detergent and subjected to sucrose gradient ultracentrifugation. The p110 DR6 form was mainly detected in the same fractions close to the top of the gradient as was CD59, a well established marker of detergent-resistant glycosphingolipid-enriched microdomains (lipid rafts) (Fig. 4A). In contrast, the p70 protein was almost exclusively present in the dense non-raft fractions at the bottom of the gradient. To evaluate the role of DR6 *S*-linked palmitoylation, wild-type DR6 and a *S*-palmitoylation-deficient DR6(C368V) mutant were ectopically expressed in HEK293 cells and the cell lysates were subjected to sucrose gradient ultracentrifugation. In contrast to endogenous DR6, only part of the transfected wild-type DR6 was localized to the light lipid raft fractions (Fig. 4B, upper panel). The distribution of the DR6(C368V) mutant in the sucrose gradient fractions did not notably differ from that of wild-type DR6, arguing for a non-essential role of Cys368 palmitoylation in the lipid raft targeting of DR6 (Fig.4B, middle panel). In addition, the treatment of NCTC cells with 2-bromopalmitate, an inhibitor of *S*-linked-palmitoylation, did not affect its localization to the light lipid raft fraction (Fig. 4C). Thus, *S*-palmitoylation of DR6 is apparently not essential for DR6 targeting to the lipid rafts.

Several recent publications point to *N*-glycosylation as a possible targeting mark of transmembrane receptors to lipid rafts [19]. In support of these findings, we also observed *N*-glycosylation-dependent targeting of transfected DR6 into lipid rafts and discovered that the localization of the *N*-glycosylation-deficient DR6(6xNQ) mutant in the light, lipid raft-containing fractions was severely reduced (Fig. 4B, lower panel). However, the treatment of NCTC cells with tunicamycin, an inhibitor of *N*-glycosylation, did not influence the localization of DR6 to the light lipid raft fractions, arguing against a potential role of *N*-glycosylation in the mobilization of DR6 into lipid rafts (Fig. 4C, lower panel). Thus, the possible role of *N*-glycosylation in the targeting of DR6 into lipid rafts remains unresolved and should be further investigated.

A recently published report proposed a potential role of DR6 in the differentiation of monocytes into immature dendritic cells [15]. The authors, using recombinant, in insect cell-produced DR6-Fc fusion protein, partially suppressed the IL-4+GM-CSF-induced expression of the differentiation marker CD1a. We used this assay for evaluating the potential role of DR6 *N*-glycosylation. Monocytes from two different donors were differentiated into immature dendritic cells, and this differentiation could be suppressed by recombinant DR6-Fc fusion protein produced in a baculoviral system (Suppl. Fig. 3A). However, using either our recombinant or other commercially available DR6-Fc fusion protein produced in HEK293FT cells, we could not reproduce inhibition of IL-4+GM-CSF-induced monocyte differentiation imposed by the DR6-Fc produced in insect cells (Suppl. Fig. 3A). Such unexpected differences between these fusion proteins prompted us to compare their properties, including their mobility in SDS-PAGE. As shown in Suppl. Figs. 3B, C the baculovirally-produced DR6-Fc, despite its 15 amino acid extension in the DR6 extracellular part, migrated mainly due to the absence of most of the *N*-linked glycosylation in SDS polyacrylamide gel faster than DR6-Fc expressed in human cells.

4. Discussion

Death receptor-6 belongs among the longest (number of amino acids) and also the least characterized receptors from the TNFR family. Apart from the common cysteine-rich domains and the membrane-proximal death domain, it also contains an unusually long linker region in its extracellular part and the intracellular C-terminal CARD-like domain [20]. In this communication we show that DR6 is also a heavily posttranslationally modified receptor and that extensive and complex *N*- and *O*-glycosylation and potentially other modifications of its extracellular part are mainly responsible for a 40 kDa mobility shift in DR6 polyacrylamide gel migration, exceptional among TNFR superfamily members. We found that both transfected and endogenous DR6 is mainly expressed in 2 forms—the major *N*- and likely *O*-glycosylated p110 protein and the minor *N*-only-glycosylated form p90, which most probably represents a DR6 precursor transiently formed mainly upon the ectopic overexpression of DR6. In contrast to the clear-cut assessment of *N*-glycosylated sites in the extracellular part of DR6, even combined enzymatic and inhibitor-based treatment targeting mucin-type *O*-glycosylation led only to an approximate 25 kDa drop in the apparent molecular weight (Fig. 2A, lane 8), pointing to either the incomplete inhibition and/or accessibility of *O*-linked oligosaccharide chains or the presence of other posttranslational modifications such as xylose-*O*-Ser-linked or *N*-acetylglucosamine-*O*-Ser-linked glycosylation.

Glycosylation of amino acids in the extracellular parts of plasma membrane proteins, including the receptors of the TNFR family, can affect the folding, trafficking, localization and function of these receptors [21]. Defects in the *N*-glycosylation of some plasma membrane receptors such as the prostaglandin E2 receptor, the α chain of FcεRI, the dopamine D5 receptor, the HLD receptor SR-BI or the TRPM8 cation channel were reported to affect their folding, trafficking to the cell surface or targeting to submembrane domains such as lipid rafts [19,22–25]. In contrast to these receptors, we found that *N*-glycosylation of DR6 is apparently not required for its plasma membrane targeting as both a *N*-glycosylation-deficient DR6 mutant and DR6 from tunicamycin-treated cells were still transported to and localized at the cell surface. Also, our *N*-glycosylation-deficient DR6-Fc fusion protein was, in contrast to the *N*-glycosylation mutant of TNFR1, transported to the cell surface of HEK293FT cells and secreted (not shown and [26]). However, *N*-glycosylation deficient DR6 still maintains a high degree of *N*-glycosylation-independent posttranslational modifications, such as *O*-glycosylation, that can perhaps compensate for the loss of hydrophilicity and ensure proper folding and transport to the cell surface. Moreover, these results clearly show that *N*-glycosylation per se is not required for these additional posttranslational modifications.

In contrast to *N*-glycosylation, which is present partially in the cysteine-rich domains of DR6, mucin-type *O*-glycosylation (and other possible posttranslational modifications in DR6 p110 protein) take place exclusively in the linking the region between CRDs and the transmembrane part. Similar linker regions containing *O*-glycosylation-rich "stalk" domains are present in some other TNFR superfamily members such as CD30, NGFR or TNFR2. *O*-linked glycosylation of serines and/or

Fig. 3. The extracellular linker region between cysteine-rich domains and the transmembrane domain of DR6 is essential for plasma membrane targeting of DR6. A. Graphical bar view of DR6 mutants; yellow—signal peptide, red—cysteine-rich domains, violet—transmembrane region, blue—death domain; DR6(C368V)—palmitoylation-deficient mutant, DR6 (6xNQ)—*N*-glycosylation-deficient mutant. B. Wild-type and mutants of DR6 were expressed in HEK293FT cells with or without tunicamycin treatment and analyzed by Western blotting. C. HeLa cells were transfected with the indicated DR6 expression plasmids, and the cellular localization of DR6 was analyzed by confocal microscopy using purified anti-DR6 (ICP) polyclonal antibodies. D. HEK293FT cells were transfected with DR6 expression plasmids, stained with anti-DR6(ECP) mAb and the DR6 cell surface expression was analyzed by flow cytometry. (red histogram represents cells stained only with the secondary antibody and the blue one cells stained with anti-DR6(ECP) mAb).

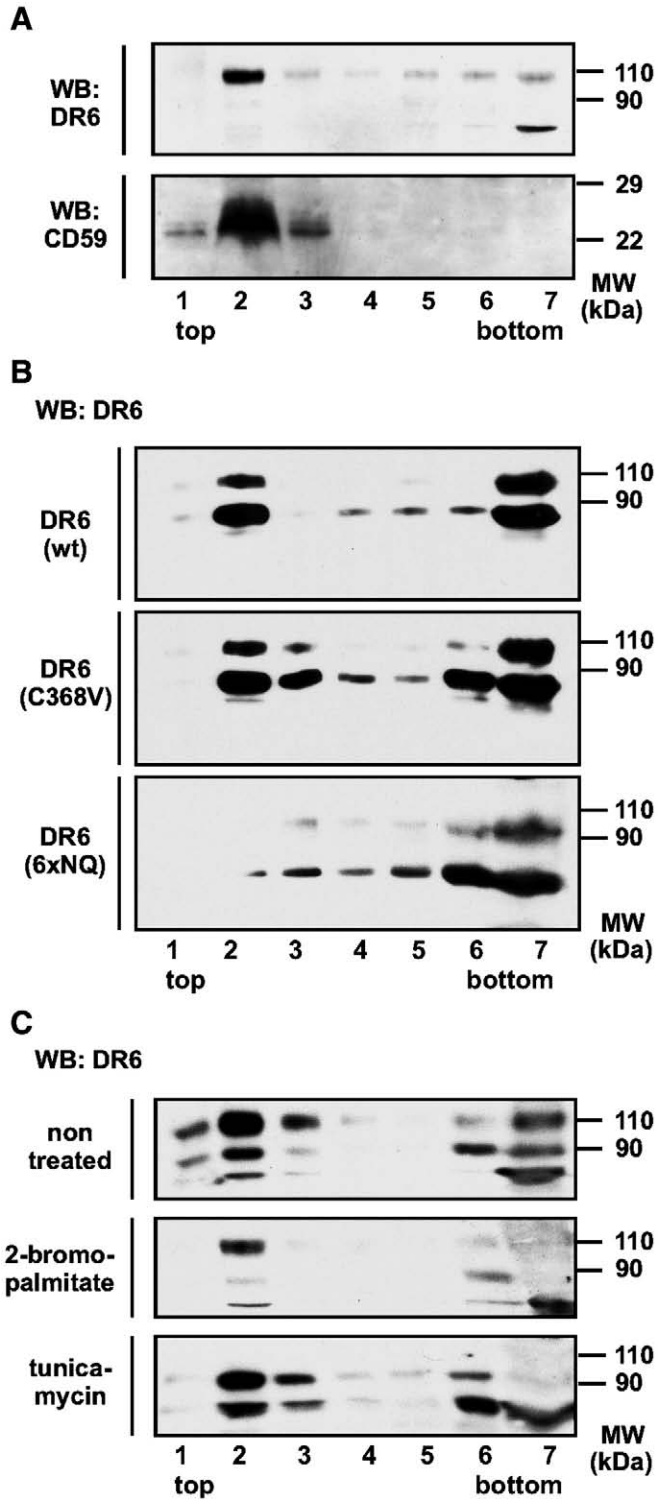


Fig. 4. *N*-glycosylation but not *S*-palmitoylation might participate in the targeting of DR6 into lipid rafts. A. NCTC cell lysate solubilized in Brij98 buffer was subjected to sucrose density gradient ultracentrifugation, and the fractions were analyzed by Western blotting using anti-DR6 or anti-CD59 (control marker of lipid rafts) antibodies. B. HEK293 cells were transfected with DR6 expression plasmids, solubilized in Brij98 buffer and analyzed as described in A. C. NCTC cells with or without 2-bromopalmitate or tunicamycin treatment were solubilized in Brij98 buffer and analyzed as described in A.

threonines in these domains could function as a pedestal for the proper orientation of CRDs towards the respective interacting ligand. In contrast to DR6, deletion of the linker region in NGFR did not affect the transport of NGFR to the plasma membrane but changed its apical-basolateral targeting in polarized cells [27,28]. Thus, the linker region might contain a sequential or structural motif required for the proper sorting of transmembrane proteins and/or, as we found for DR6, for their effective transport to the cell surface. DR6 lacking the entire linker region most probably remains stacked in the Golgi system.

In addition to plasma membrane targeting and localization, the glycosylation of DR6 can potentially regulate the interaction of DR6 with its recently described ligands APP or APLP2 [13]. Glycosylation was shown to both attenuate the interaction of FGFR1 with FGF-2 [29] and to enhance the ligand binding of the 5-HT3A receptor [30]. We also found apparent functional differences between insect and human cell-produced DR6-Fc proteins in respect to modulation of monocyte differentiation to dendritic cells. This discrepancy could be related to evident differences in *N*-linked glycosylation of the recombinant receptors produced in insect versus human cells (Suppl. Fig. 3C), hinting either at the functional significance of DR6 glycosylation status requirements for interaction with its ligand (possibly APLP2, which is expressed in human CD14+ monocytes, data from GNF Symatlas at http://symatlas.gnf.org/SymAtlas/) or at a non-specific off-target effect of the insect cell-produced, and possibly differently folded DR6-Fc. Thus, the role of posttranslational modifications in the interaction between DR6 and its ligand/-s remains to be explored.

An additional posttranslational modification present in Fas/CD95 and TRAIL-R1, but not in TNFR1 or TRAIL-R2, is *S*-palmitoylation of the membrane-proximal cysteine(s), which is apparently required for their association with lipid rafts and effective pro-apoptotic signaling [17,18]. We proved that similarly as these death receptors, DR6 is *S*-palmitoylated at the membrane-proximal Cys368 residue in the intracellular region. In cells expressing high levels of DR6 such as NCTC and PC3, the majority of DR6 is associated with the light, Brij98-insoluble (but NP40-soluble) lipid raft-containing membrane fraction. However, neither the treatment of cells with a palmitoylation inhibitor nor the usage of a DR6 point mutant lacking *S*-palmitoylation affected the subcellular localization of DR6, suggesting that other motifs might participate in DR6 targeting to Brij98-insoluble lipid rafts. Among possible lipid raft-targeting mechanisms could be the death domain-mediated targeting shown for TNFR1, the extracellular glycosphingolipid-binding motif found in Fas/CD95 or *N*-linked glycosylation-assisted targeting as shown for the TRPM8 cation channel [19,31,32]. Our *N*-glycosylation-deficient mutant of DR6 was inefficiently targeted to lipid raft fractions, suggesting a positive effect of *N*-glycosylation on DR6 localization to lipid rafts. However, as the inhibition of endogenous DR6 *N*-glycosylation in NCTC cells by tunicamycin did not significantly affect the distribution of DR6 in the lipid raft fractions, we cannot unambiguously attribute to the *N*-glycosylation of DR6 its lipid raft-targeting mark and surely other, yet unknown concurrent mechanisms can take over, when the N-linked glycosylation of DR6 is disabled in e.g. tunicamycin-stressed cells. Nevertheless, the lipid raft localization of DR6 could be important for the regulation and outcome of DR6 signaling. The lipid raft localization of TNFR1 was proven to be essential for the activation of NFκB, p42MAPK or RhoA signaling in a cell-specific manner [33–35].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:. 10.1016/j.bbamcr.2009.07.008.

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