Biological and Structural Characterization of Glycosylation on Ephrin-A1, a Preferred Ligand for EphA2 Receptor Tyrosine Kinase*

Sara Ferluga1, Roy Hantgan5, Yehuda Goldgur5, Juha P. Himanen5, Dimitar B. Nikolov5, and Waldemar Debinski1†

From the 1Department of Neurosurgery, Brain Tumor Center of Excellence, Comprehensive Cancer Center and the 5Department of Biochemistry and Molecular Medicine, Wake Forest School of Medicine, Wake Forest University, Winston-Salem, North Carolina 27157 and the 5Structural Biology Program, Memorial Sloan Kettering Cancer Center, New York, New York 10065

Background: Ephrin-A1 is the preferred ligand for EphA2 receptor.

Results: Biological assays and crystal structure analysis document that ephrin-A1 deglycosylation abrogates ligand's binding and receptor activation, and also protein folding and cellular localization.

Conclusion: The glycosylation of ephrin-A1 enables EphA2 receptor binding and activation by stabilizing Eph/ephrin heterotetramers.

Significance: The glycosylation of ephrin-A1 is indispensable for the protein biological activity.

The EphA2 receptor tyrosine kinase is overexpressed in a number of malignancies and is activated by ephrin ligands, most commonly by ephrin-A1. The crystal structure of the ligand-receptor complex revealed a glycosylation on the Asn-26 of ephrin-A1. Here we report for the first time the significance of the glycosylation in the biology of EphA2 and ephrin-A1. Ephrin-A1 was enzymatically deglycosylated, and its activity was evaluated in several assays using glioblastoma (GBM) cells and recombinant EphA2. We found that deglycosylated ephrin-A1 does not efficiently induce EphA2 receptor internalization and degradation, and does not activate the downstream signaling pathways involved in cell migration and proliferation. Data obtained by surface plasmon resonance confirms that deglycosylated ephrin-A1 does not bind EphA2 with high affinity. Mutations in the glycosylation site on ephrin-A1 result in protein aggregation and mislocalization. Analysis of Eph/ephrin crystal structures reveals an interaction between the ligand’s carbohydrates and two residues of EphA2: Asp-78 and Lys-136. These findings suggest that the glycosylation on ephrin-A1 plays a critical role in the binding and activation of the EphA2 receptor.

Ephrin-A1 (eA1)2 is a high affinity ligand for the cell surface transmembrane receptor EphA2. Eph receptors, with 16 known members, belong to the receptor tyrosine kinases (RTK)s family playing critical roles in many physiological processes, including embryonic tissue patterning, neuronal development, angiogenesis, and tumorigenesis (1–4). Ephrins and Eph receptors are divided into two subclasses, A and B, and with some exceptions, eAs bind EphA-type receptors and ephrin-Bs (eBs) bind EphBs. The Eph classification is based on the specificity of ligand interaction and sequence homology of the extracellular domain (5), whereas eAs differ from eBs in the manner in which they are anchored to the plasma membrane: glycosylphosphatidylinositol (GPI)-linkage (eA1s–5) or transmembrane domain (eBs1–3) (1, 2). The binding between ephrins and Eph receptors on neighboring cells triggers bidirectional signals: forward signals in the Eph-expressing cells dependent on the receptor kinase activity, and reverse Src-family kinase-dependent signals on the ephrin-expressing cells (6, 7).

Eph receptors are type-I transmembrane proteins with a highly conserved N-terminal ligand binding domain (LBD), a cysteine-rich region and two fibronectin type III repeats, involved in receptor dimerization, on the extracellular side. The intracellular portion contains a juxtamembrane region, a kinase domain, a sterile α-motif domain and a PDZ binding motif (8, 9). The recently determined eA1/EphA2 crystal structure reveals a “lock-and-key” type binding mechanism dominated mainly by Van der Waals contacts and with a 1:1 binding stoichiometry. The binding affinity is mostly derived by a loop of 15 conserved amino acids connecting the G and H β-sheets (G-H loop) on eA1 which is inserted into a channel on the surface of EphA2 (10). Upon ligand binding, EphA2 undergoes tyrosine autophosphorylation followed by internalization and degradation (11). We have recently performed detailed structure-function relationship analysis using Ala scanning mutagenesis of the G-H loop, which confirmed this loop being critical for the binding of eA1 to the EphA2 receptor. Several point mutants showed increased affinity for the receptor, including Q109A, T115A, and G117A (12).

Membrane-bound oligomerized eA1 was historically proposed to be necessary to activate EphA2 by inducing receptor clustering and subsequent internalization and down-regulation (13–16). However, as our laboratory recently demonstrated, eA1 wild-type (eA1-WT) full-length (aa. 1–205) is a GPI-anchored cell membrane protein that, on cell surface, is cleaved by...
several metalloproteinases like MMP-1,-2,-9, and -13 (17), and released as a soluble fully active monomeric protein (12, 18).

Crystallographic studies revealed that eA1 is glycosylated indeed (10). Here, for the first time, we document the importance of this glycosylation on eA1 not only for protein production and localization, but also for proper receptor activation and down-regulation.

EXPERIMENTAL PROCEDURES

Cell Culture and Cell Transfection—GBM cell line U-251 MG was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and has been authenticated by Idexx Radil (Columbia, MO). MDA MB 321 breast cancer and PC-3 prostate cell lines were obtained from the ATCC and were grown in the ATCC-recommended media. U-251 MG cells were grown in DMEM medium containing 10% (v/v) FBS and 0.1 mmol/liter non-essential amino acids. U251-MG [eA1](+/-) cells were grown in the above medium with 200 µg/ml geniticin. Mutant eA1 plasmids were transfected into U-251 MG cells at ~60% confluency in Opti-MEM (Invitrogen, Carlsbad, CA) using Lipofectamine 2000 (Invitrogen) following the instructions of the supplier. After overnight incubation at 37 °C, Opti-MEM media was replaced with regular growth medium containing 20% FBS. Antibiotic selection (geneticin, 800 µg/ml) was added to regular growth medium 48 h after transfection. After 5–6 days of selection, individual clones were isolated with cloning rings, transferred into a 24-well plate, and maintained in growth medium with 200 µg/ml geneticin. Mutant eA1 expression was verified by Western blot and high-expressing clones were further cultured.

Enzymatic Protein Deglycosylation and Tunicamycin Treatment—U-251 MG [eA1](+/-) CM and the recombinant human eA1-WT, monomeric and dimeric and the recombinant mouse eA1-Fc and human eA5-Fc (R&D System, Minneapolis, MN), were enzymatically deglycosylated with the enzymes PNGase F and Endo H (New England Biolabs, Ipswich, MA). CM and native recombinant glycoproteins were deglycosylated overnight at 37 °C according to the manufacturer’s instructions with 1U/µg of enzyme. Deglycosylated recombinant proteins were additionally purified by affinity chromatography to remove the enzyme. U-251 MG [eA1](+) cells at ~80% confluency were treated with 1 µg/ml tunicamycin (MP Biomedicals, Solon, OH) for 24 h at 37 °C. Cell lysates and media were analyzed by Western blot.

E1A Mutagenesis—E1A mutants were generated using Phusion Site-directed Mutagenesis Kit (Thermo Scientific). E1A-WT cloned in pcDNA3.1(+) vector (12) was used as template for mutagenesis. The following forward primers: 5'-GTC-CTCTGCTGGGCGATTTCAATTCCCAAG-3'; 5'-GTC-CTCTGCGGAATTTCTCAACCTCAGAAG-3'; and 5'-GTC-TTCTGCGGAATTTCTCAACCTCAGAAG-3', were used to create eA1-N26A, -N26Q, and -N26D, respectively. The reverse primer was 5'-GGTGCTGGCGATCAGCAGCGGG-3'. Each clone was sequenced (Wake Forest University DNA Sequencing Laboratory), amplified using Escherichia coli DH5α cells and transfected in U-251 MG cells as above described.

Production of Recombinant eA1-WT and Mutants—Recombinant eA1-WT (aa. 19–182) and mutants (eA1-N26A, -N26Q, and -N26D) were produced both in the monomeric (N-terminal 6× histidine tag) and in the dimeric form (C-terminal Fc tag) in the Baculovirus expression system (BD Biosciences, San Diego, CA). EA1-WT and mutants were amplified by PCR from the previously produced clones using the primers: 5'-TATA-GGATCCCATACCCATACCATTGA GTGCGCCACACC-GTC-3' (forward, 6×His, BamHI), for the monomeric form, 5'-ATATATTGATTCGATCGCCACCCGTC-3' (reverse, EcoRI). The amplified fragments were cloned into BamHI-EcoRI sites in the Baculovirus transfer vector pAcGP67-B (BD Biosciences) and sequenced. SF9 insect cells were co-transfected as previously described (12). SF9-Baculovirus infected serum-free media containing the recombinant proteins were collected, filtered, and stored at 4 °C until purification.

Protein Purification—Monomeric eA1-WT and mutants were purified by Nickel affinity chromatography (HisTrap HP affinity column, GE Healthcare, Piscataway, NJ). SF9 serum-free media containing the recombinant proteins were dialyzed with 100:1 buffer to sample volume ratio in 20 mM sodium phosphate buffer with 0.5 M NaCl and 20 mM imidazole at pH 7.4. Dialysis buffer was exchanged twice over a period of ~24 h. After dialysis, pH was adjusted to 7.4, and samples were filtered through a 0.22-µm pore filter. The column was equilibrated with 10 column volumes of dialysis buffer. The chromatography was performed using a step gradient and recombinant proteins were eluted at 50% elution buffer (20 mM sodium phosphate buffer with 0.5 M NaCl and 500 mM imidazole, pH 7.4). Imidazole was removed by buffer exchange with centrifugal filter devices (10K Amicon Ultra-4, Millipore), and purified filtered proteins were stored in PBS at ~80 °C.

Dimeric eA1-WT and mutants were purified by Protein G affinity chromatography (HiTrap Protein G HP, GE Healthcare) as recommended by the supplier. SF9-Baculovirus-infected media with the recombinant proteins were filtered as previously described and loaded into the Protein G column. After protein purification, pH was adjusted, and buffer was exchanged as before. Purified filtered proteins were stored in PBS at ~80 °C. Recombinant proteins purity was evaluated on 10 or 12% Coomassie-stained SDS-PAGE.

Western Blots—Western blotting was performed as previously described (12). Primary antibodies used included rabbit eA1 (V-18) polyclonal antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), mouse EphA2 monoclonal (1:1000, EMD Millopore Corp., Billerica, MA), β-actin (1:50000, Sigma). Anti-rabbit and anti-mouse secondary antibodies (Sigma) were used 1:5000. Films were scanned at a resolution of 600 dpi using a HP ScanJet3979 and Adobe Photoshop 5.0 Software. Immunoreactive bands were quantified by Scion Image Corporation software (National Institutes of Health, Bethesda, MD) and normalized to the NT sample.

U-251 MG Cell Rounding and EphA2 Down-regulation Assays—1.5 × 10^5 U-251 MG cells were plated in 60-mm dishes and grown over-night at 37 °C, 5% CO₂. The next day, cells were treated with different concentrations of monomeric or dimeric recombinant eA1. Cell rounding was observed ~20 min after protein administration. Treated cells were incu-
bated for 4 h before lysing the cells to check EphA2 degradation. Cell lysates were prepared as previously described (12). U-251 MG [eA1](+)-WT and mutants cells were stained by immunofluorescence.

**Immunofluorescence Staining**—U-251 MG [eA1](+)-WT and mutants cells were grown overnight on sterile glass slides. Slides were then washed twice in PBS and cells were fixed for 3 min in acetone at −20 °C. After two more washes in PBS, slides were either used or stored at −80 °C. U-251 MG cells were grown overnight on sterile glass slides and then treated with eA1 (1 μg/ml) in the appropriate medium. After ~15 min, cell rounding reaction was stopped, and cells were fixed by adding 10% buffered formalin. After fixation slides were washed twice in PBS and blocked for 1 h in 10% normal goat serum (NGS) at room temperature. EA1 (Santa Cruz Biotechnology, 1:500) and EphA2 (EMD Millipore Corp., 1:1000) primary antibodies were diluted in 1.5% NGS and incubated overnight at 4 °C. Slides were washed at least three times, 10 min each, in PBS, and incubated 2 h with secondary antibody and Nuclear Counterstain (DAPI, 1:1000) at room temperature in the dark. Slides were washed three times for 10 min each in PBS and mounted with fluoroguard. For Phalloidin staining, cells were permeabilized in PBS + 0.1% Triton X-100 after formalin fixation, stained with AlexaFluor 488 phalloidin (1:200, Molecular Probes, Inc.) and DAPI for 2 h, rinsed, and mounted with fluoroguard. Photomicrographs were taken with a 60× lens.

**Anchorage-independent Growth Assay**—U-251 MG cells (2 × 10^3) were plated in 6-well plates in growth medium plus 0.35% BactoTMagar (BD), on a base layer of growth medium plus 0.5% agar. Monomeric and dimeric eA1, both glycosylated and enzymatically deglycosylated, were added to the growth media. Untreated U-251 MG cells were grown under the same conditions as control. Fresh media containing the different forms of eA1 were added to the cells 3 days and 1 week after plating. Colonies were counted after 14 days. Clusters of colonies greater than 25 cells were counted in 10 random fields at low power. Each experimental point was done in triplicate for each assay.

**Migration Assay**—Wounds were made in a confluent monolayer of U-251 MG cells plated in 6-well plates with a sterile 200 μl tip. Monomeric eA1, both glycosylated and deglycosylated, was added to the media at the concentrations of 1 and 6 μg/ml. Untreated U-251 MG cells were used as control. Phase contrast microscopy pictures of the same field were taken at 12 and 24 h after scratching. Wounds width was measured in μm in five different places, at 12 h time point, for each of the two wounds for each treatment, using ImagePro Plus software. Percentage of wound closure was calculated for graphical representation.

**Surface Plasmon Resonance**—Binding interactions between immobilized EphA2 and recombinant eA1-WT, both in the monomeric and in the dimeric form, as well as the corresponding deglycosylated proteins, were measured by SPR in a Biacore T100 instrument as previously described (12). Kinetic profiles were obtained with each ephrin construct in duplicate experi-

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**FIGURE 1.** The glycosylation consensus sequence on eA1 is highly conserved among the eAs. Alignment of human eA1 protein sequence (N-terminal ~60 amino acids) to the protein sequences of human eAs and eBs available in the database (GenBank™, NCBI).

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**FIGURE 2.** In vitro eA1 deglycosylation. A, Western blot of eA1 from U-251 MG [eA1](+)-CM treated with PNGase F and Endo H. B, immunodetection of eA1 in cell lysates and media of U-251 MG [eA1](+)-cells grown in the presence of tunicamycin. C, SDS-PAGE of monomeric and dimeric human eA1-WT glycosylated and PNGase F deglycosylated. D, Western blot analysis of EphA2 in U-251 MG, MDA-MB-231, and PC-3 cells after treatment with eA1 or Deg-eA1.
ments at 300, 1000, and 3000 nM using EphA2 covalently cou-
pled to a CM5 chip to achieve a sparse monolayer (5300 
response units, RU) in the sample channel and a blank immo-
obilization in the reference channel. Binding was measured for 
700 s followed by a 1500 s dissociation step; time-dependent RU 
changes obtained with buffer delivery were subtracted from 
each protein trace to obtain double-corrected kinetic profiles. 
The maximum binding RU signals obtained at each ephrin con-
centration were used to measure the affinity of monomeric and 
dimeric eA1, WT and deglycosylated, for EphA2 by fitting the 
data to single-site saturable model (12).

Dynamic Light Scattering—Protein size distributions for 
recombinant eA1-WT (aa. 19–182) and mutants (eA1-N26A, 
-N26Q, and -N26D), both in the monomeric and in the dimeric 
form, were measured by DLS in a Malvern Nano-S Zetasizer 
(Malvern Instruments, Worcestershire, UK) as previously 
described (36). The intensity-weighted distributions of hydro-
dynamic diameters were converted to volume % distributions 
to minimize the contributions of small quantities of aggregated 
proteins (37).

Ephrins Alignment—The protein sequences of the hu-
mans ephrins were downloaded from GenBank™ database 
(UCSC) with the following accession numbers: AAH95432.1, 
EAW69517.1, AAH17722.1, AA107484.1, AAH75054.1 for 
eA1, eA2, eA3, eA4, and eA5, respectively; and AAH16649.1, 
AAH74857.1, AAH42944.1 for eB1, eB2, and eB3, respectively. 
Protein alignment was analyzed by ClustalW2 (EBI). Protein 
glycosylation was predicted using NetNGlyc 1.0 Server (CBS 
Prediction Servers).

Statistical Analysis—Data are expressed as means ± S.D. as a 
result of at least three independent experiments. Probability (p) 
values were calculated using the ANOVA one-way test lead by 
MS Excel; p values <0.05 were considered to be statistically 
significant.
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**RESULTS**

*eA1 Glycosylation Site Is Highly Conserved Among eAs—Human eA1 (GenBank™ AAH95432.1) is predicted to be glycosylated on Asn-26 (NetNGlyc 1.0 Server), and that was confirmed by the crystal structure of the EphA2/eA1 complex (19). To analyze the evolutionary conservation of the glycosylation site on eA1, the N-terminal region of the protein (first 38 amino acids) was aligned to the protein sequences of human eAs and eBs available in the database (GenBank™, NCBI) (Fig. 1). The glycosylation consensus sequence (NXS) in eA1 (*pink highlighted*, Fig. 1) is highly conserved in the human eAs as well as the surrounding region (*yellow highlighted*, Fig. 1), and all eAs are predicted to be glycosylated on this consensus sequence. Among the human eBs, only eB2 is predicted to be glycosylated on the conserved consensus sequence (Fig. 1).

*eA1 Glycan Removal and Inhibition of eA1 Glycosylation—Conditioned media (CM) from stable human eA1-expressing U-251 MG cells (18) were treated with the enzyme PNGase F under non-denaturing conditions. PNGase F removes N-linked glycans from glycoproteins cleaving between the innermost GlcNAc (*N*-acetyl-D-glucosamine) and Asn residues of high mannose, hybrid, and complex oligosaccharides (20, 21). The immunoreactive eA1 showed a significant reduction in the molecular mass of the protein after PNGase F treatment compared with the non-treated CM (Fig. 2A). The same result was obtained by treating the CM with Endo H under the same conditions (Fig. 2A). Endo H cleaves with more specificity the chitoiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins (20, 22), thus indicating that eA1 glycan has to belong to one of these last two groups of oligosaccharides.

To further confirm the glycosylation on eA1, U-251 MG [eA1](+/-) cells were grown in the presence of tunicamycin to block the synthesis of all N-linked glycoproteins (23). As tunicamycin is known to causes cell cycle arrest in G1 phase (24), we observed a reduction in the amount of total eA1 in the cell lysates, which was produced in non-glycosylated form (Non Gly-eA1) in accordance with the smaller size of the protein (Fig. 2B). Non-glycosylated eA1 was no longer detected in the media of tunicamycin-treated cells, in sharp contrast to eA1-WT (Fig. 2B), demonstrating that N-glycosylation is critical for proper eA1 folding and/or release.

Deglycosylated Recombinant Monomeric and Dimeric eA1 Lose Biological Functions—To investigate possible changes in functionality of Deg-eA1, first we enzymatically deglycosylated monomeric (N-terminal 6×His tag) and dimeric (C-terminal Fc tag) forms of the ligand. The proteins were additionally purified to remove the enzyme PNGase F (Fig. 2C). Soluble monomeric eA1 was previously shown to be active in inducing EphA2 receptor internalization and down-regulation (4, 12, 18). Monomeric Deg-eA1 was tested on GBM, breast and prostate cancer cell lines overexpressing EphA2 (25, 26). EphA2 was no longer down-regulated 4 h after treatment with Deg-eA1 to the degree observed with eA1-WT in all three cell lines tested (Fig. 2D).

Furthermore, U-251 MG cells were treated with 1 µg/ml of glycosylated and Deg-eA1 on slides and either phalloidin or EphA2 immunofluorescent stained. No morphological changes and no EphA2 internalization were observed after administration of Deg-eA1, in sharp contrast to eA1-WT (Fig. 3A).

Next, we explored the ability of deglycosylated monomeric and dimeric eA1 to elicit cell rounding of U-251 MG GBM cells (18, 27). Unlike eA1-WT, Deg-eA1 did not round the cells within 1 h of treatment in either monomeric or dimeric form (data not shown). In addition, while EphA2 was down-regulated 4 h after treatment with glycosylated monomeric or dimeric eA1, the degree of down-regulation was reduced after treatment with deglycosylated ligands (Fig. 3, B and C).
EA1 demonstrates sequence and structure similarities with eA5 (19). Therefore, we deglycosylated human recombinant dimeric eA5 and tested on GBM cells. Contrary to Deg-eA1-Fc, Deg-eA5-Fc was still very active in inducing cell rounding (Fig. 3D) and EphA2 receptor down-regulation (Fig. 3E).

Deg-eA1 Does Not Inhibit Either Migration or Proliferation of GBM Cells—We have previously shown that a stable ectopic eA1 expression in U-251 MG cells leads to a significant defect in both cell migration and proliferation compared with parental cells (18). The same effect has been observed when glioblastoma cells were cultured in the presence of recombinant eA1-WT monomers and dimers delivered to immobilized EphA2 (18, 28). In the cell migration assay, Deg-eA1 lost the ability to inhibit wound closure on U-251 MG cells (Fig. 4A). Since the experimentally calculated doubling time for these cells was $\sim 22$ h, the measurements were taken at 12 h after scratching. Deg-eA1 demonstrated little activity in inhibiting anchorage-independent growth (Fig. 4B). The results again strongly indicate that eA1 glycosylation is indispensable for the protein biological activity.

Deglycosylation Compromises eA1 Binding to the EphA2 Receptor—In an attempt to explore the reason why Deg-eA1 lost its biological activity, we performed detailed analysis of ligand binding to the EphA2 receptor. Data obtained by surface plasmon resonance (SPR) demonstrated that recombinant eA1-WT (aa. 19–182), both in the monomeric and in the dimeric form, exhibited high-affinity binding to immobilized EphA2 (Fig. 5A). Fitting the data from duplicate experiments to a single site, saturable model yielded $K_d = 330 \pm 29 \text{ nM}$; $B_{\text{max}} = 132 \pm 3$ RU. Data obtained with deglycosylated proteins (monomer, squares; dimer, circles) were fit by linear regression to obtain the solid line. C, SPR binding signals obtained in duplicate experiments with WT and deglycosylated proteins at 1000 nM. D, SPR signals obtained as a function of time with recombinant eA1-WT monomers (dashed line) and dimers (dashed-dot line), each at 1000 nM, delivered to immobilized EphA2 demonstrate rapid, reversible binding. Kinetic traces obtained with deglycosylated proteins (monomer, solid line; dimer, short-dashed line) show smaller signal changes.
eA1 Glycosylation Stabilizes the Ligand-Receptor Heterotramer in the EphA2/eA1 Crystal Structures—The crystal structures of the EphA2 ectodomain (ECD) bound to eA1 and eA5 suggested a structural basis for the formation of Eph/ephrin signaling clusters at the interfaces of interacting cells (19). Specifically, these signaling clusters nucleate from high-affinity Eph/ephrin heterodimers, which assemble into heterotetramers that further aggregate into higher-order oligomers. Analysis of the available EphA2/eA1 and EphA2/eA5 structures (10) reveals that, in all cases, the carbohydrate moiety attached at ephrin position 26 interacts with the ligand-binding domain of an EphA2 (Fig. 6, A–C).

In light of these observations, we also revisited the previously published structure of the complex between the EphA2 ligand-binding domain (LBD) and eA1 (10), which did not include the Asn-26 attached carbohydrate in the originally published model. After careful refinement, four sugar rings could be clearly seen attached to Asn-26 of eA1 and were built into the structure (Fig. 6D). These align precisely with the conformation of the carbohydrates observed in the full ECD EphA2/ephrin structures, and also extend to less than 4 Å from the EphA2 Asp-78 and Lys-136 residues (Fig. 6D). However, there are noticeable differences between eA1 and eA5 that need to be further investigated. There are eight EphA2/eA1 heterodimers in the asymmetric unit and similar electron density from the sugars can be observed in all of these complexes (Fig. 6E).

Thus, the crystallographic studies of the receptor/ligand complexes clearly support a role for eA1 glycosylation in stabilizing the EphA2/eA1 heterotetramers and thus in EphA2-mediated signaling.

eA1 Glycosylation Site Mutants Are Impaired in Secretion and Function—We generated three eA1 non-glycosylated mutants by exchanging Asn-26 with an Ala, a Gln and an Asp. For each mutant three different clones were immunostained for eA1 displaying a similar profile (data not shown). U-251 MG [eA1-N26A], U-251 MG [eA1-N26Q], and U-251 MG [eA1-N26D] cells produced eA1, which was mainly present in the cell lysates (Fig. 7A) and much less in the media (Fig. 7B). EA1 immunofluorescent staining of cells transfected with eA1-WT and eA1-N26A, -N26Q, -N26D mutants demonstrated the localization of the proteins mainly in the perinuclear region while the cells were also of elongated morphology in striking contrast to eA1-WT-transfected cells (Fig. 7C). However, significantly more of eA1-N26D was detected in the media compared with the other mutants (Fig. 7D). Thus, CM of U-251 MG [eA1-N26D] cells were concentrated and immunoreactivity was quantified by densitometry and compared with eA1-WT CM (Fig. 7D). The same amounts of eA1-WT CM and eA1-N26D concentrated CM were added to U-251 MG cells. E1-A1-N26D CM failed to induce cell rounding (data not shown) and EphA2 down-regulation on U-251 MG cells, in contrast to eA1-WT CM (Fig. 7E).
Deglycosylation Does Not Alter eA1 Structural Integrity While Glycosylation Site Mutagenesis Results in Misfolded Proteins—Recombinant eA1 mutants (eA1-N26A, -N26Q, and -N26D) (aa. 19–182) were produced in the Baculovirus protein expression system both in monomeric and dimeric forms, to force the secretion of the protein into the media. The purified fractions were analyzed by dynamic light scattering (DLS) to study the size of the molecules compared with eA1-WT, both dimeric and monomeric, glycosylated, and deglycosylated.

Size distribution data for eA1 yielded peaks at 5.1 nm for both the WT protein and its deglycosylated form (Fig. 8A). Dimeric eA1-Fc-WT exhibited a peak at 10.2 nm; here deglycosylation resulted in a small downward shift with a peak at 9.1 nm. The 5-nm peak is consistent with the ~4 nm diameter calculated treating eA1-WT monomer as spherical, hydrated 21-kDa protein (29), and the absence of material at larger sizes suggests that eA1, both WT and deglycosylated forms, are free of higher-order oligomers and aggregates. A similar argument can be made for dimeric 81-kDa eA1-Fc-WT where increased hydration and asymmetry can explain the difference between the calculated 6.5 nm diameter and the observed 9–10 nm peaks. In contrast to these results, DLS data obtained with the eA1-N26A, -N26Q, and -N26D mutants, as well as the corresponding eA1-Fc mutants, all showed multimodal distributions at larger diameters indicative of substantial aggregation consistent with improper protein folding (Fig. 8, B and C).

**DISCUSSION**

In this work, we present for the first time clear-cut evidence for the critical role of glycosylation in the biology of eA1. The eA1 glycosylation consensus sequence is highly conserved among the eAs but not among the eBs. Interestingly, the role of ephrin glycosylation in stabilization of the Eph/ephrin tetramers is clearly visible in the EphA2/eA1 structure (Fig. 6), but not in the structures containing EphA4 (30), suggesting that this might possibly be an EphA2-specific characteristic. This required further investigation using different members of the Eph/ephrin families, as also suggested by the activity of Deg-
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![Graph A](image1.png)  
**Figure 8.** *Non glycosylated eA1 recombinant mutants aggregate in solution.*  
A, size distribution data obtained by DLS with recombinant eA1-WT monomers and dimers, as well as the deglycosylated forms demonstrate the absence of aggregated species. B, size distribution data with recombinant monomeric eA1 mutants demonstrate the presence of multiple aggregated species for eA1-N26A, eA1-N26Q, and eA1-N26D. C, size distribution data with recombinant eA1-Fc mutants demonstrate the presence of multiple aggregated species for eA1-Fc-N26A, eA1-Fc-N26Q, and eA1-Fc-N26D.

In eukaryotes there are three major classes of N-linked saccharides: high-mannose, hybrid, and complex oligosaccharides (32). In our experiments, eA1 was enzymatically deglycosylated by both PNGase F and Endo H enzymes. Both enzymes remove N-linked glycans from glycoproteins however, PNGase F cleaves between the GlcNAc and the Asn residue of all classes of oligosaccharides while Endo H cleaves with more specificity at the level of the chitobiose core of only high mannose and some hybrid oligosaccharides. Our data suggest that the eA1 glycan belongs to one of these two classes of oligosaccharides as Endo H was active in removing the glycan. Importantly, deglycosylation occurred without disrupting protein folding and did not result in protein aggregation. In contrast, tunicamycin treatment or mutation of the essential Asn residue resulted in loss of eA1, likely due to degradation of misfolded protein, underscoring the importance of this N-glycan for proper eA1 folding and export.

A number of studies have shown the EphA2 receptor operating as a tumor suppressor when stimulated with eA1 (1). The glycosylation on eA1 was first documented by Himanen *et al.* discussing the crystal structure of the eA1/EphA2 complex (10). Our current findings demonstrate how the glycosylation on eA1 is critical to ensure receptor internalization and down-regulation. Deg-eA1 tested on GBM, breast, and prostate cancer cell lines indicated the absence of downstream signaling pathways (12, 17, 18). 

**AA1** acts also as a membrane-bound protein that oligomerizes and activates EphA2 by inducing clustering of the receptor and subsequent internalization and degradation (13–16), hence we performed parallel experiments using dimeric eA1 (as a fusion protein with the IgG-Fc). Our results demonstrate loss of biological activity of Deg-eA1 both in the monomeric and dimeric forms. We show, using surface plasmon resonance spectroscopy, that this is a consequence of an impaired binding of Deg-eA1 to the EphA2 receptor.

Involvement of ephrin glycosylation in the formation of the EphB2/eB2 2:2 tetramers was previously suggested based on the location of the glycosylation site near the tetramerization interface (8). However, as bacterially produced non-glycosylated eB2 bound the receptor with high affinity and specificity (33), the possible involvement of the saccharide in the binding was discarded.

When we attempted to produce eA1 in a prokaryotic expression system, the protein was insoluble and, after purification under denaturing conditions, the refolding did not take place (data not shown). These data are in accordance with our results showing non-glycosylated eA1-N26A, -N26Q and -N26D full-length mutants, expressed in U-251 MG GBM cells, localized in the perinuclear region of the cell and much less in the media, supporting the hypothesis of misfolding and aggregation likely in the endoplasmic reticulum. Moreover, DLS analysis of secreted recombinant monomeric and dimeric eA1-N26A-Q-D-purified mutants, produced in the Baculovirus expression system, confirmed the presence of large particles in the sample compatible with protein aggregates. On the other hand, enzymatic deglycosylation of the properly folded eA1-WT resulted in a soluble, aggregate-free protein. Thus, it appears that eA1 glycosylation is also essential for the correct folding of the protein (34).

**Targeting the EphA2 receptor with eA1-based therapeutics** has been proposed to be a promising approach based on the tumor-suppressing activity exerted by the ligand on the receptor (26). Indeed, eA1 has been conjugated to *Pseudomonas* exotoxin A (PE38) (25, 35), to gold-coated silica nanoshells (36) and on the surface of albumin microspheres (37). In this context, understanding the role and importance of eA1 glycosylation, combined with the ability to improve the binding affinity via G-H loop mutagenesis (12), and to protect eA1 from proteolytic cleavage, will aid the rational design of new therapeutics targeting EphA2.

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