

N-Glycosylation as determinant of epidermal growth factor receptor conformation in membranes

Karol Kaszuba^{a,1}, Michał Grzybek^{b,c,1}, Adam Orłowski^a, Reinis Danne^a, Tomasz Róg^a, Kai Simons^{d,2}, Ünal Coskun^{b,c,2}, and Ilpo Vattulainen^{a,e,2}

^aDepartment of Physics, Tampere University of Technology, FI-33101 Tampere, Finland; ^bPaul Langerhans Institute Dresden of the Helmholtz Centre Munich at the University Clinic Carl Gustav Carus, TU Dresden, 01307 Dresden, Germany; ^cGerman Center for Diabetes Research (DZD e.V.), 85764 Neuherberg, Germany; ^dMax Planck Institute for Molecular Cell Biology and Genetics, 01307 Dresden, Germany; and ^eCenter for Biomembrane Physics (MEMPHYS), University of Southern Denmark, 5230 Odense, Denmark

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The epidermal growth factor receptor (EGFR) regulates several critical cellular processes and is an important target for cancer therapy. In lieu of a crystallographic structure of the complete receptor, atomistic molecular dynamics (MD) simulations have recently shown that they can excel in studies of the full-length receptor. Here we present atomistic MD simulations of the monomeric N-glycosylated human EGFR in biomimetic lipid bilayers that are, in parallel, also used for the reconstitution of full-length receptors. This combination enabled us to experimentally validate our simulations, using ligand binding assays and antibodies to monitor the conformational properties of the receptor reconstituted into membranes. We find that N-glycosylation is a critical determinant of EGFR conformation, and specifically the orientation of the EGFR ectodomain relative to the membrane. In the absence of a structure for full-length, posttranslationally modified membrane receptors, our approach offers new means to structurally define and experimentally validate functional properties of cell surface receptors in biomimetic membrane environments.

EGFR | lipids | MD simulation | lipid-protein interaction | proteoliposomes

Receptor tyrosine kinases (RTKs) are cell surface receptors that receive and transduce signals mediating a variety of critical cellular processes, including cell growth, migration, proliferation, differentiation, and apoptosis. Among the many RTKs, the most studied is the epidermal growth factor receptor (EGFR), not least because of its involvement in the development and progression of epidermoid cancers and its resulting importance as a target for antineoplastic therapies.

Structurally, the EGFR consists of the ectodomain (ECD) (further subdivided into four subdomains, DI–IV), the transmembrane domain (TMD), and the intracellular tyrosine kinase domain (TKD). Ligand binding induces conformational transitions of the ECD that stabilize receptor dimerization, culminating in the activation of the intracellular TKD and subsequent propagation of the activation signal (1). To prevent receptor activation and signaling in the absence of ligand, the structurally tethered ECD of monomeric EGFR blocks the intrinsic capacity of the TMD and the intracellular TKD to dimerize (2). Ligand binding is believed to release the self-inhibitory tether and facilitate receptor oligomerization and activation (3–6). A detailed understanding of the structural regulation of the intact full-length receptors in their native membranes promises to reveal the molecular basis for receptor regulation (7); however, the methodological limitations associated with crystallizing transmembrane proteins, together with the high flexibility of the full-length receptor, have prevented high-resolution crystallographic analysis.

To fill this gap, extensive atom-scale molecular dynamics (MD) simulations were recently performed to elucidate the structural dynamics of the EGFR in a two-component lipid bilayer (8). These studies suggest a large interfacial contact area between the membrane and the ecto- and intracellular domains of the unliganded monomeric and dimeric receptors. However, experimental

data at the single-molecule level stipulate a greater distance between the ECD of the receptor and the membrane in the absence of ligand (9, 10).

To resolve this discrepancy, the present study considers two key parameters that were not included in previous simulations of the monomeric EGFR: N-glycosylation of the ectodomain of EGFR that contributes up to 50 kDa of the total molecular weight of ~178 kDa (11, 12), and a lipid environment that prevents the EGFR from ligand-independent, and thus aberrant, activation (13). Comparing glycosylated and nonglycosylated EGFR, we find that N-glycosylation is critical for the conformational arrangement of the ECD subdomains DI–IV and their interfacial contact area with the membrane. In our simulations, only the N-glycosylated receptor adopts a conformation that is in good agreement with previous experimental FRET studies for the distance between the EGFR ECD and the membrane (9, 10). Experimental ligand and antibody binding assays on EGFR proteoliposomes provide validation of the MD simulations.

Results

To follow the dynamics of membrane-embedded monomeric EGFR, we have performed extensive atomistic MD simulations of the near-full-length receptor [lacking the unstructured C-terminal

Significance

Structural analysis of growth factor receptors in their membrane environment is key for understanding their functions that are vital to the development and survival of organisms. High structural flexibility and posttranslational modifications of the full-length receptors, however, hinder structural analysis at high resolution. Here, we used atomistic molecular dynamics simulations and biochemical experiments with proteoliposomes to elucidate the role of N-glycosylation with regard to the structural properties of the human epidermal growth factor receptor (EGFR). We find that N-glycosylation critically determines membrane interactions and structural arrangement of the ligand-binding EGFR ectodomain. This combined approach provides new means to structurally explore and experimentally validate functional properties of cell surface receptors and test therapeutic agents, such as monoclonal antibodies.

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¹K.K. and M.G. contributed equally to this work.

²To whom correspondence may be addressed. Email: simons@mpi-cbg.de, coskun@plid.de, or ilpo.vattulainen@tut.fi.

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tail of the kinase domain, as in previous MD simulations (8)]. *N*-glycosylation is required for EGFR trafficking, efficient ligand binding, and receptor activation (12, 14–17), suggesting this modification confers an additional informational level to the underlying polypeptide structure (18), required for achieving a functional receptor state. Although number, sequence, size of branches, and fucosylation of glycans vary between specific proteins and cell types, Man₃GlcNAc₂ residues constitute the cell type-independent minimal core for all *N*-glycans attached to proteins, critical for protein folding in the endoplasmic reticulum (19, 20). Here, we compared the receptor conformation either lacking or containing Man₃GlcNAc₂ glycan residues at 10 well-described positions of the ectodomain (21, 22) (*SI Appendix, Fig. S1*).

The receptor was embedded in a membrane composed of a ternary lipid mixture of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), *N*-stearoyl-D-erythro-sphingophosphorylcholine (SM), and cholesterol, a plasma membrane mimetic composition that recapitulates as closely as possible the experimental conditions for biochemical reconstitution of the EGFR in proteoliposomes (*SI Appendix, Table S1*) (13). In synthetic membranes, similar ternary lipid compositions give rise to two immiscible fluid membrane phases: a DOPC-enriched liquid-disordered and a cholesterol/SM-enriched liquid-ordered phase (23). Using this lipid composition for EGFR proteoliposomes, we previously showed that the presence of both phases is critical to prevent ligand-independent kinase activation, whereas membranes with low cholesterol levels allowed ligand-independent receptor activation (13), in agreement with aberrant EGFR activation in cellular membranes on cholesterol depletion (24).

Atomistic simulations of membrane receptors are challenging because the time scales of conformational changes are relatively slow, and therefore adequate sampling of receptor conformation

is an important concern. In this work, we have accounted for this issue by carrying out a number of complementary simulations. For both the glycosylated and the nonglycosylated receptors, the MD simulations covered 1,000 ns (together these constitute “simulation 1”; for PDB files, please see *Datasets S1* and *S2*). To improve sampling, we next started from different (independent) initial conditions and repeated 1,000-ns runs for both the glycosylated and the nonglycosylated EGFR (“simulation 2”; for PDB files, please see *Datasets S3* and *S4*). In addition, the intermediate configurations of the first simulations at 300 and 1,000 ns were used to consider how the glycosylated EGFR evolves toward the nonglycosylated structure after the removal of glycans.

Conformational Arrangement of the EGFR Ectodomain. In all configurations (nonglycosylated and glycosylated), the ECD of the EGFR underwent large-scale conformational changes within the first 500 ns (Fig. 1) as a consequence of the hinge-bending domain motions at two different regions of the ECD (*SI Appendix, Fig. S2*).

The first of the identified hinges (H1) was formed by a short loop (amino acid residues 615–621) connecting the ECD to the TMD of the receptor. The second hinge (H2) encompassed the residues 230–239 and 259–261 of subdomain DII. Within the first nanoseconds, the ECD bending motion around hinge H1 resulted in the formation of a large contact interface area between subdomain DIV and the membrane, in all configurations (Fig. 1 *C* and *D*). In the nonglycosylated receptor, hinge H2 caused a rotation of subdomain DI toward the membrane. Once stably attached, subdomain DI buried ~1,000–2,000 Å² of the solvent-accessible surface area (SASA) in the interface with lipids (Fig. 1 *B* and *D*).

The presence of Man₃GlcNAc₂ glycan residues significantly altered the relative arrangement of the individual receptor subdomains and their alignment on the membrane (Fig. 1, *SI Appendix, Fig. S3*, and *Movies S1* and *S2*). The glycan moieties attached at positions

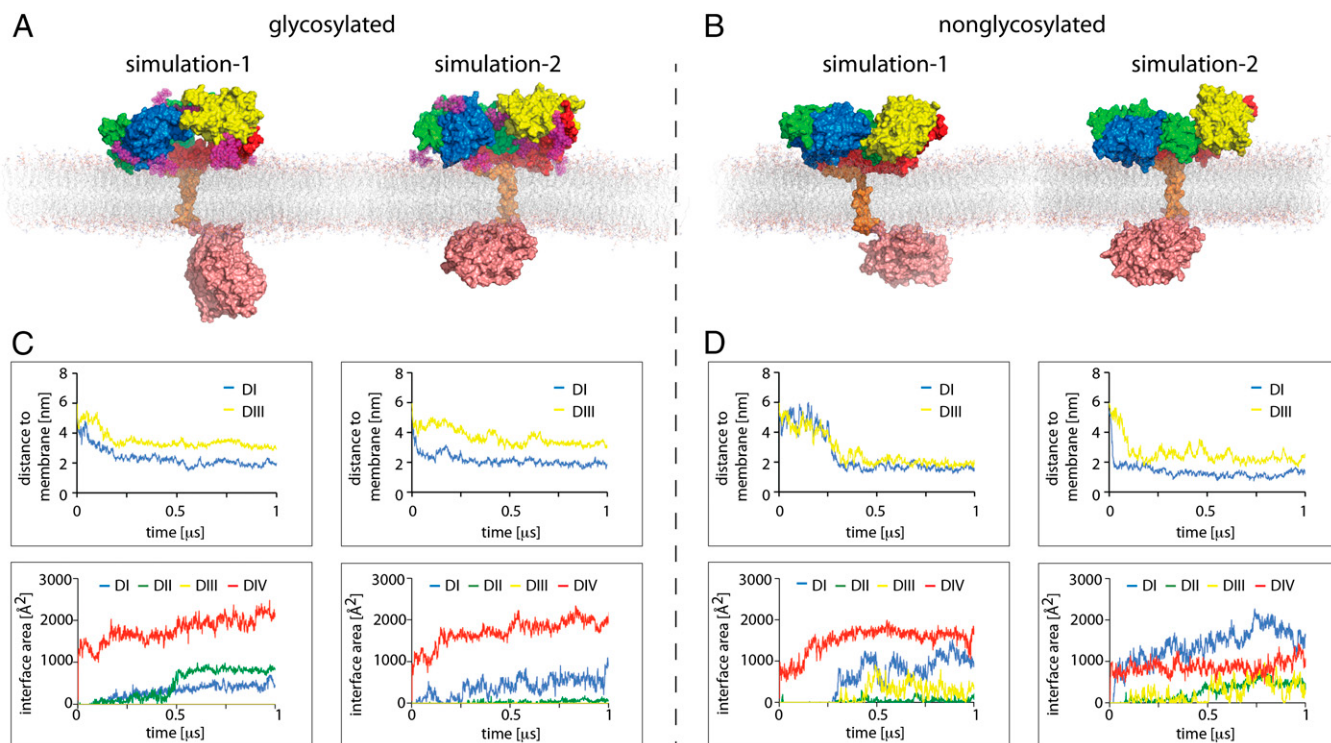


Fig. 1. The endpoint structures (1,000 ns) of the (A) glycosylated and (B) nonglycosylated EGFR in DOPC/SM/cholesterol membranes. The receptor structure is color-coded throughout the panels as follows: subdomains DI (blue), DII (green), DIII (yellow), and DIV (red); TM domain (orange); intracellular TKD (salmon); glycans (purple). (C and D) The distances of the center of mass of subdomains DI and DIII to the membrane (*Upper*) and membrane interface area of ECD subdomains DI–DIV (*Bottom*), for (C) glycosylated and (D) nonglycosylated EGFR. For corresponding PDB files, see *Datasets S1–S4*.

glycosylation patterns are cell type-specific, whereas 2E9 inhibits low-affinity EGFRs in various cell types (29, 31, 32), as well as the recombinant insect SF+ cell protein product in our experiments. Overall, our data support the view that DIII is the primary ligand-binding site, and the 2E9 antibody inhibits low-affinity EGF binding through preferential interactions with DI.

Conformational Coupling Across the Membrane. The EGFR transmembrane domain has been previously described to be critically involved in the communication between the ECD and the intracellular TKD. Therefore, the possible regulatory function of the TMD has received substantial attention (2, 8, 33–38). Two GxxxG dimerization motifs present on both ends of the TMD are supposed to regulate the association of ligand-free and ligand-bound dimers (2, 8). In our MD simulations, we did not observe glycan-based structural changes in the C-terminal dimerization motif (⁶³⁷ALGIG⁶⁴¹) (*SI Appendix, Fig. S6 A and B*), whereas the N-terminal motif (⁶²⁵GMVGA⁶²⁹) was flexible and eventually even unfolded in nonglycosylated EGFR simulation 1.

N-terminal TMD dimerization is also required as a regulatory step for asymmetric dimer formation, and hence activation of the intracellular TKD, a process guided through membrane proximal, intracellular juxtamembrane segments A and B (JM-A and JM-B). Upon N-terminal TMD dimerization, the JM-A segments interact with the membrane and form an α -helical, anti-parallel homo-dimer (8, 39, 40). The α -helical structure of the JM-A dimer has been confirmed in NMR and MD simulation studies of TMD–JM-A fragments (2, 8, 41) and in MD simulation of the active EGFR dimer (8).

In both of our simulations of the nonglycosylated receptor, the JM-A segment is primarily α -helical (Fig. 3). Interestingly, the helical property of the JM-A segment strikingly correlates with the formation of a rapidly evolving and stable contact interface area of the TKD with the membrane (Fig. 3*B*, *SI Appendix, Fig. S7*, and *Movie S2*). In the glycosylated EGFR, this feature can be observed in simulation 2 only. In simulation 1, the TKD does not form a large membrane contact interface with the same kinetics, although its membrane interactions are stable and increase gradually (*SI Appendix, Fig. S7*, and *Movie S1*). As a consequence, the initially α -helical JM-A segment unfolds promptly at the beginning and remains unfolded throughout the entire simulation. We also observe that the membrane interaction of the TKD is not restricted to a preferred orientation.

Discussion

High-performance atomistic MD simulations of the full-length EGFR embedded in membranes in combination with experimental reconstitution systems are a promising approach to bridge the gap between biochemistry and classical structural methods. With this multipronged approach, we have evaluated the effect of *N*-glycosylation of the EGFR in a defined lipid environment that promotes ligand-dependent EGFR activation.

Unique among posttranslational modifications, glycosylation is immensely diverse, with different cell lines exhibiting distinct patterns of protein glycosylation, largely as a result of the expression of varying repertoires of glycosidases and glycosyltransferases (20). Hence, the sugar sequence, number and size of branches, and fucosylation of additional glycans vary between specific proteins and cell types.

The heterogeneity, considerable size, and flexibility of glycan residues hinder protein crystallization. For the human EGFR, among other proteins, this problem can be bypassed by recombinant production of the receptor in insect cells (27, 42, 43) in which glycosylation sites, ligand binding, and receptor activation properties are not compromised, although glycan branching and branch length are significantly reduced. Alternatively, the receptor is expressed in mammalian cells and then partially enzymatically

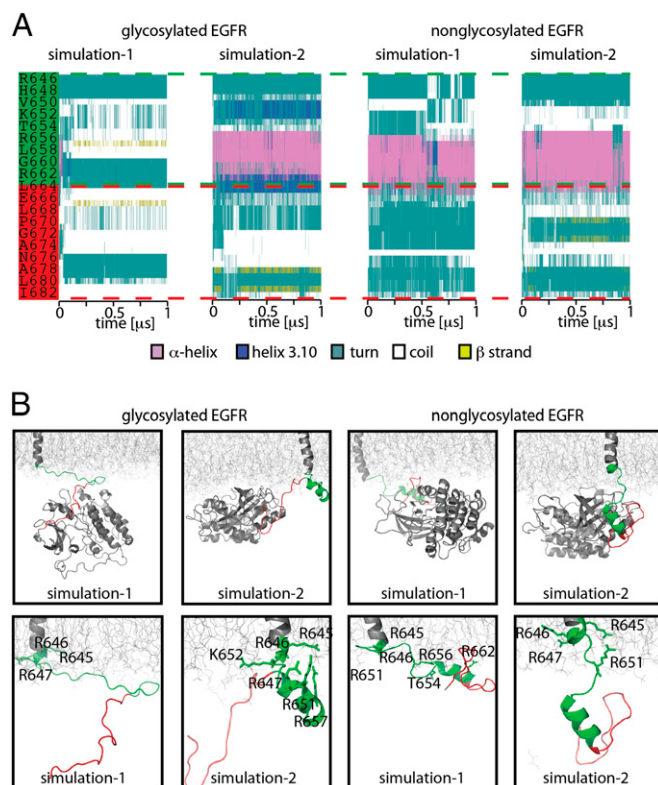


Fig. 3. (A) Secondary structure changes of the intracellular JM-A and JM-B segments, color-coded in green and red, respectively. (B) The structure of the EGFR intracellular domain and hydrogen bonding between JM-A and lipids at the 1,000 ns endpoints of the respective simulations. Amino acid residues that interact with lipids for more than 50% of the simulation time are highlighted as stick representation. Corresponding residue occupancy times can be found in *SI Appendix, Tables S3 and S4*.

deglycosylated before crystallization (4, 5). The resulting polypeptide retains some *N*-glycosylation residues, possibly because they are structurally inaccessible to enzymatic cleavage in the folded receptor complex. However, even when present, glycan structures are only partially resolved in crystal structures of the EGFR ectodomain (4, 5, 27, 42, 43). Therefore, the structural consequences of glycosylation, and the resulting functional effects on receptor regulation are difficult to assess.

On the basis of our MD simulations and experiments, glycosylation critically determines the structural arrangement of the ECD, and in particular, of the ligand-binding domains DI and DIII. Our data confirm the notion that DIII is the major site of ligand binding, whereas DI has only a minor contribution (30, 44). Additionally, DIII is known to bind the ligand when expressed as soluble protein (25) or upon proteolytic cleavage of the ECD (45); antibodies and nanobodies that bind to DIII [C225 (cetuximab), IMC11F8, VHH 7D12] compete with EGF for the binding site on this subdomain (27, 42, 46).

To further interrogate the credibility of the simulated structures, we compared the computed protein membrane distances to the previously reported simulations of the monomeric EGFR (8), as well as to experimental data (9, 10). Although our simulations of the nonglycosylated receptor are in good agreement with the previous simulations (8), significant differences in the arrangement of the ECD subdomains DI and DIII are observed for the glycosylated receptor (*SI Appendix, Table S2*). Here, the presence of glycans propelled DI, and especially DIII, away from the membrane, up to ~1.9 and 3.2 nm, respectively (the distance computed along the membrane normal from the center of mass

of the ECD subdomain to the average position of phosphorous atoms in lipid headgroups) (Fig. 1C and *SI Appendix, Table S2*). FRET studies with N-terminally fluorescence-tagged EGFR suggested that the distance between the fluorescent label and the membrane ranges from ~6.2 to 8 nm (9, 10). This is in discrepancy with the simulations of the nonglycosylated receptor, where the N terminus of the receptor is located ~3.1–3.5 nm away from the membrane (*SI Appendix, Table S2*). In comparison, the N-terminal distance from the membrane for the glycosylated receptor is 4.5 nm. Assuming that a fluorescent dye or probe would contribute additionally at least 1 nm, the total distance would increase to 5.5 nm, which is in much better agreement with the published experimental data of 6.2–6.4 nm and 8 nm for the acyl carrier protein (9) and YFP-tagged EGFR (10), respectively. The difference between simulations and the experimental single-molecule data is still discernible, but is expected since we have considered the Man₃GlcNAc₂ core glycosylation only, accounting for about 30% of total glycans of the EGFR expressed in mammalian cells. Moreover, the plasma membrane also contains glycolipids known to interact with the EGFR ECD (13, 47, 48), which may also increase the observed distances at the cellular level.

Critically, however, for our understanding of receptor activation, the coupling mechanism between the ECD and the intracellular kinase domain across the biological membrane remains ambiguous. Very recently, Arkhipov et al. presented MD simulations of the ligand-stabilized and glycosylated human EGFR dimer that lacks the intracellular juxtamembrane and kinase domains (49). Compared with their previous simulations of the liganded, but nonglycosylated full-length EGFR dimer (8), the ECD now significantly interacts with the membrane. Truncation of the ectodomains, in contrast, leads to aberrant dimerization and activation of the EGFR kinase domain (2). The ill-defined linkage mechanism between ligand binding and stabilization of the active kinase domains stems, at least partially, from methodological limitations resulting from solubilized receptors being studied in detergent micelles, augmenting flexibility of the membrane proximal sequences; that is, the kinase domains of ligand-bound dimeric receptors can adopt flexible conformations (50) that correlate with the active or inactive state of EGFR dimers (51). It is therefore tempting to conclude that the membrane itself holds the key for understanding domain coupling (7).

Eukaryotic cells tune the composition of their membranes through directed lipid sorting along the secretory pathway and selective lipid transport across the bilayer. As a consequence, the physicochemical properties of their membranes vary significantly throughout the cell (52), exemplified by the plasma membrane being highly enriched in cholesterol (~35–40 mol%) and sphingolipids, both critically regulating membrane fluidity and thickness (53). Upon cholesterol depletion, the EGFR kinase domain is activated in a ligand-independent manner (24, 54). A very similar effect was observed in synthetic reconstitution experiments: ligand-dependent EGFR activation was observed only in bilayers with high cholesterol and sphingomyelin levels, while reconstitution of EGFR into bilayers enriched in low melting temperature phosphatidylcholine led to aberrant receptor activation (13). Previous MD simulations and NMR studies have not accounted for the compositional and physicochemical particularities of the plasma membrane. Occasionally used synthetic lipids such as 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine and 1,2-distearoyl-*sn*-glycero-3-phosphocholine do have high transition temperatures (41 °C and 55 °C), but these do not mimic the fluidity of natural membranes in the absence of cholesterol. In contrast, short-chain lipids such as 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine form thin and homogenous bilayers, and therefore are expected to energetically constrain the TMD, as described for synthetic transmembrane helices in membranes of varying thickness (55).

For our MD simulations, we have used a lipid composition that is as close as possible to the ternary lipid mixture that was used in the reconstitution experiments, which are themselves a reasonable (although dramatically simplified) mimic of the three key lipid types (saturated, unsaturated, cholesterol) present in live cell plasma membranes. The advantage of this approach is that it provides mutual validation between computational and biochemical experiments. Although the MD simulations of the glycosylated receptor lead to a highly reproducible structural arrangement of the EGFR ECD on the membrane, the properties of the juxtamembrane fragments and orientation of the TKD are flexible throughout all simulated systems. In the MD simulations of Arkhipov et al. (8), the TKD and JM-A fragment are attracted to the membrane by the presence of negatively charged phosphatidylserine. In our systems, the TKD and JM-A fragment still are capable of interacting with the membrane (Fig. 3 and *SI Appendix, Fig. S7, Tables S3 and S4*), although it contains neutral lipids only. In contrast to the well defined ECD arrangement, the intracellular modules interact with the membrane without any preferred orientation. The presence of negatively charged lipids could possibly change this into a guided process. Moreover, the stability of the α -helical JM-A segment seems to depend on a critical threshold of the membrane contact area interface of the entire TKD, rather than the direct interactions of this fragment with the lipids. Unlike the other simulations, in simulation 1 of the glycosylated receptor, JM-A unfolds promptly and remains unfolded throughout the simulation, which correlates with the limited formation of the membrane interface. This finding, together with the previously described electrostatic membrane interactions with lipids, highlights the capacity of the intracellular JM-A domain to sense and interact with various lipid environments, possibly in an electrostatic switch-like mechanism (6, 8, 56, 57). It should also be noted that the orientation of the TKD and the helicity of the JM-A segment do not seem to have an influence on the properties of the kinase active site of the TKD. Independent of glycosylation state or membrane association, the TKD retains the previously described and characterized features of the nonactive conformation, with the activation loop inserted in the kinase active site and the inactive orientation of the helix α C (39, 51) (*SI Appendix, Fig. S8*).

In summary, glycosylation of the EGFR appears to be critical for the conformational arrangement of the ECD and its membrane contact sites, whereas the influence on TMD properties, JM-A segment, and the orientation and membrane association of the TKD are not significantly affected. Our approach to study the full-length, posttranslationally modified EGFR by high-performance MD simulations supplemented with proteoliposomal reconstitution systems has the potential to become a comprehensive toolbox for studying membrane proteins in biomimetic lipid environments.

Materials and Methods

Reconstruction of EGFR Receptor Structure for Atomistic MD Simulations. A nearly full-length receptor (2–994) that lacks only the very C-terminal auto-phosphorylation tail (residues 995–1,186) was reconstructed from separately crystallized PDB structures: 1YY9 (27) for the extracellular domain and 2RGP/3GOP (40, 51) structures for the intracellular TK domain. The structure of the TM helix was built de novo. The fully reconstructed EGFR chain was subsequently embedded in a ternary lipid mixture (DOPC/SM/cholesterol). The lipid compositions of these systems were designed to match the previously reported in vitro reconstitution conditions (13). The receptor was N-glycosylated in silico with core Man₃GlcNAc₂ residues at documented sites (4, 5, 21, 22, 27, 42, 43).

We performed atomistic MD simulations for the glycosylated and the nonglycosylated receptor. Both systems were simulated for 1,000 ns. These studies are referred to in this work as simulation 1. In addition, we improved sampling by carrying out simulation 2, in which we repeated the 1,000-ns simulations for the glycosylated and nonglycosylated receptors by starting from different (independent) initial conditions. The results of these replicas were largely consistent with those of simulation 1, as discussed earlier (*SI Appendix*). A more detailed description of system construction, simulation conditions, additional simulation data as well as experimental methods are provided in *SI Appendix*.

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