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In-depth biophysical analysis of interactions between therapeutic antibodies and the extracellular domain of the epidermal growth factor receptor

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ABSTRACT

Targeting of the epidermal growth factor receptor (EGFR) with monoclonal antibodies has become an established antitumor strategy in clinical use or in late stages of drug development. The mAbs effector mechanisms have been widely analyzed based on in vivo or cell studies. Hereby we intend to complement these functional studies by investigating the mAb–EGFR interactions on a molecular level. Surface plasmon resonance, isothermal titration calorimetry, and static light scattering were employed to characterize the interactions of matuzumab, cetuximab, and panitumumab with the extracellular soluble form ecEGFR. The kinetic and thermodynamic determinants dissected the differences in mAbs binding mechanism toward ecEGFR. The quantitative stoichiometric data clearly demonstrated the bivalent binding of the mAbs to two ecEGFR molecules. Our results complement earlier studies on simultaneous binding of cetuximab and matuzumab. The antibodies retain their bivalent binding mode achieving a 1:2:1 complex formation. Interestingly the binding parameters remain nearly constant for the individual antibodies in this ternary assembly. In contrast the binding of panitumumab is almost exclusive either by directly blocking the accessibility for the second antibody or by negative allosteric modulation. Overall we provide a comprehensive biophysical dataset on binding parameters, the complex assembly, and relative epitope accessibility for therapeutic anti-EGFR antibodies.

The epidermal growth factor receptor (EGFR)[1] is aberrantly activated in a variety of epithelial tumors—colon, breast, lung, head, and neck—and is a target in anticancer therapy. Aberrant EGFR activation has been observed as a result of receptor overexpression, gene amplification, activating mutations, overexpression of associated ligands, and/or loss of negative regulatory controls [1]. EGFR-dependent signaling activates proliferation, protection from apoptosis, loss of differentiation, migration, and invasion—all known hallmarks of cancer. Moreover, increased EGFR expression has been correlated to poor clinical prognosis for patients [2]. Therapeutic strategies targeting this receptor include highly specific monoclonal antibodies (mAbs) that are currently used and tested in the clinic [3]. These antibodies inhibit EGFR signaling by means of binding to the easily accessible extracellular domain of the receptor and thereby hindering ligand-dependent receptor dimerization required for intracellular tyrosine kinase autotransphosphorylation [3–5]. In order to highlight the molecular basis behind antibody-mediated EGFR inhibition, crystal structures have been solved for the antigen-binding fragments (Fabs) of different inhibitory mAbs in complex with the extracellular regions of EGFR [5,6]. These structural studies have contributed to a clarification of the different EGFR binding modes and deduced mechanisms of receptor inhibition. The inhibitory effect of the antibodies cetuximab [7], IMC-11F8 [8], and zalutumumab [9] has been related mainly to direct occlusion of the ligand-binding site. Epitope mapping studies have shown that panitumumab and cetuximab bind to a comparable surface-exposed region of the receptor [10]. Therefore, panitumumab probably also acts via a direct ligand-binding site occlusion. On the other hand inhibition by matuzumab is believed to occur due to steric hindrance of the open conformation required for high affinity ligand binding and dimerization [11]. Earlier work of Kamat et al. also indicate that cetuximab and the murine variant of matuzumab, mAB425, bind to distinct epitopes as they can associate to soluble EGFR simultaneously [12]. However detailed data on complex

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[1] *Abbreviations used:* ecEGFR, extracellular soluble form of epidermal growth factor receptor; EDC, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide; EGFR, epidermal growth factor receptor; Fabs, antigen-binding fragments; ITC, isothermal titration calorimetry; NHS, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; SEC, size-exclusion chromatography; SLS, static light scattering; SPR, surface plasmon resonance.

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stoichiometry and possible mutual modulation of antibody binding have not been published. All these mAbs additionally inhibit EGFR activation by stabilizing a tethered receptor conformation, which is dimerization incapable [5].

This study was focused on further biophysical characterization of mAb complexes with the extracellular soluble form of EGFR (ecEGFR) in terms of assembly state, kinetics, and thermodynamics of binding. The biophysical methods surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), and static light scattering (SLS) were applied. SPR-based biosensors like Biacore have been widely used to determine binding kinetics and affinity constants of antibody–receptor interactions in real time by measuring the binding of soluble receptor domains to the immobilized antibody on a sensor chip [13–16]. The study of binding kinetics is essential to fully understand the molecular interactions and how they impact on the biological processes as by the drug target residence time [17]. Such information is unavailable from equilibrium data that focus more on the affinity within the complex [18].

The thermodynamic characterization of an interaction complements structural and kinetic information by providing information on the molecular forces that lead to complex formation [19–21]. The molecular binding forces can be dissected into enthalpic (ΔH) and entropic (ΔS) contributions. The enthalpy term is related to the strength of directed interactions (H-bonds, van der Waals) that take place in the complex in relation to that in the solvent [22,23]. The entropy term is related to changes in molecular degrees of freedom, solvent organization, and phenomena of conformational dynamics such as folding microstates of proteins [24,25]. Favorable (positive) entropy changes are often associated with the release of water molecules from a binding interface whereas unfavorable (negative) entropy values are often linked to conformational or dynamic restrictions [26,27]. For biomolecular interactions the binding enthalpy and in turn the entropic factors have been found to be largely temperature dependent and this has been associated with the solvation changes in the binding interface. A descriptive parameter for the burial of binding interfaces from solvating water molecules is the heat capacity ΔC_P. The balance of polar and apolar surface area in the buried interface is modulating the sign and magnitude of the ΔC_P value. However this simplification is only valid for rigid body interactions. Significant restrictions of conformational or dynamic variability during the complex formation do also impact on the ΔC_P parameter and result into indicative largely negative values [28]. The ITC is the standard method for direct determination of binding thermodynamics [29] and it delivers as well the affinity and stoichiometry of binding. However measuring the temperature dependence of binding affinities provides an avenue also for SPR to determine binding enthalpies exploiting the advantages of this technique in terms of low protein consumption. Herein we will provide and compare thermodynamic data on the ecEGFR interactions generated with both methodologies.

The molecular size of complexes formed in solution can be analyzed by SLS. By this the complex assembly and stoichiometry of binding can be deduced [30]. As the mAb–ecEGFR complex is stable enough (slow dissociation), the monomeric species, heterodimers, and heterotrimers can be resolved by size-exclusion chromatography (SEC) prior to SLS analysis, as seen in several reports [14,31,32].

For the present biophysical characterization we selected the therapeutic antibodies matuzumab, cetuximab, and panitumumab that cover a broad range of EGFR binding affinities, with K_D values between 10^{-8} and 10^{-10} M [7,11,33]. In terms of the complex assembly state, the mAbs are found to bind bivalently to ecEGFR and the consequent formation of mAb-mediated ecEGFR dimers is demonstrated. Kinetic and thermodynamic characterization of complex formation provides reasons for the different mAb–ecEGFR affinities. Furthermore, studies of the interdependent and simultaneous binding of mAb combinations to ecEGFR delivered insights into allosterism and relative epitope accessibility.

Material and methods

Materials

An extracellular soluble form of EGFR comprising residues 25–640 and an additional 17 residues obtained from PD Dr. Wolfgang Weber, Universitätsklinikum Hamburg-Eppendorf (Hamburg, Germany) was used for this work [34,35]. The monoclonal antibodies matuzumab and cetuximab were provided by Merck KGaA (Darmstadt, Germany). Panitumumab was commercially available. In all cases, purities greater than 95% have been observed by SDS-PAGE for mAb samples (data not shown). Fab fragments were generated by enzymatic cleavage with papain digestion and further purified by protein A affinity chromatography using the Pierce Fab Preparation Kit from Thermo Scientific (Rockford, IL, USA). Completion of digestion into Fab fragment was confirmed by SDS–PAGE. EGF was from US Biological (Swampscott, MS, USA) and TGF-α was from Chemicon/Millipore (Billerica, MA, USA). Protein A for surface plasmon resonance biosensor immobilization was obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden).

Total protein concentration of stock protein solutions was determined by UV measurements at 280 nm (data not shown). Extinction coefficients for ecEGFR, EGF, TGF-α, matuzumab, and cetuximab were estimated by the Edelhoch method [36]. For panitumumab a primary sequence was not available; thus protein concentration was taken from the manufacturer label and cross-confirmed by a Bradford protein assay.

SPR kinetic analysis

SPR studies were carried out using a Biacore T100 biosensor from GE Healthcare Bio-Sciences AB (Uppsala, Sweden) [37,38]. The CM5 sensor chips (research grade), amine coupling reagents (0.05 M *N*-hydroxysuccinimide (NHS), 0.2 M *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC), 1 M ethanolamine HCl [pH 8.5], 10 mM sodium acetate buffer [pH 4.5 and 5.0]), and regeneration reagents (10 mM glycine HCl [pH 1.5 and 2.0], 50 mM NaOH) were also obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). SPR data were collected with Biacore T100 Control Software and analyzed using Biacore T100 Evaluation Software, both delivered with the instrument. All experiments were done using PBS buffer with 3 mM EDTA, 0.05% (v/v) Tween 20 [pH 7.4]. Amine coupling was used to directly immobilize proteins onto Biacore CM5-chips from GE Healthcare (Uppsala, Sweden). The kinetics of mAb–ecEGFR binding was studied using two alternative assay designs. The first assay design involved direct immobilization of protein A (100 μg/mL) in 10 mM sodium acetate [pH 4.5] for 7 min with a final immobilization level of 4800 response units (RU). Anti-EGFR antibodies matuzumab, cetuximab, or panitumumab (0.5 μg/mL) were captured onto this protein A surface for 30 s with capture levels of 60 RU. ecEGFR solutions covering a concentration range 1.6–800 nM were flown over the thus captured antibodies. Protein A surfaces were regenerated with a 30 s pulse of 10 mM glycine [pH 1.7]. The second assay design for mAb–ecEGFR kinetic studies involved direct immobilization of ecEGFR (1.7 μg/mL) in 10 mM sodium acetate [pH 5.0] for 6.7 min with a final immobilization level of 470 RU. Solutions of matuzumab, cetuximab, or panitumumab Fab fragments covering a concentration range 1.6–800 nM were flown over the thus immobilized ecEGFR. ecEGFR surfaces were regenerated with a 15 s pulse of 10 mM NaOH and 1 M NaCl solution. All kinetic studies were performed at a flow rate of 40 μl/min with an association