THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 29, Issue of July 16, pp. 30375–30384, 2004

Printed in U.S.A.

Identification of the Epitope for the Epidermal Growth Factor Receptor-specific Monoclonal Antibody 806 Reveals That It Preferentially Recognizes an Untethered Form of the Receptor*

Received for publication, February 4, 2004, and in revised form, April 8, 2004
Published, JBC Papers in Press, April 9, 2004, DOI 10.1074/jbc.M401218200

Terrance G. Johns‡§¶, Timothy E. Adams§‖, Jennifer R. Cochran§, Nathan E Hall‡‡, Peter A. Hoyne‖, Mark J. Olsen**, Yong-Sung Kim**, Julie Rothacker‡‡, Edouard C. Nice‡‡, Francesca Walker‡‡, Gerd Ritter§§, Achim A. Jungbluth§§, Lloyd J. Old§§, Colin W. Ward‖, Antony W. Burgess‡‡, K. Dane Wittrup**, and Andrew M. Scott‡**

*From the ‡Tumour Targeting Program, Ludwig Institute for Cancer Research, Austin Hospital, Heidelberg 3084, Australia, ‖CSIRO Health Sciences and Nutrition, Parkville 3052, Australia, **Chemical Engineering & Bioengineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, ‡‡Epithelial Biochemistry Laboratory, Ludwig Institute for Cancer Research, Royal Melbourne Hospital, Parkville 3050, Australia, and §§Ludwig Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center, New York, New York 10021*

The epidermal growth factor receptor (EGFR) is overexpressed in many epithelial cancers, an observation often correlated with poor clinical outcome. Overexpression of the EGFR is commonly caused by *EGFR* gene amplification and is sometimes associated with expression of a variant EGFR (de2–7 EGFR or EGFRvIII) bearing an internal deletion in its extracellular domain. Monoclonal antibody (mAb) 806 is a novel EGFR antibody with significant antitumor activity that recognizes both the de2–7 EGFR and a subset of the wild type (wt) EGFR when overexpressed but does not bind the wt EGFR expressed in normal tissues. Despite only binding to a low proportion of the wt EGFR expressed in A431 tumor cells (~10%), mAb 806 displays robust antitumor activity against A431 xenografts grown in nude mice. To elucidate the mechanism leading to its unique specificity and mode of antitumor activity, we have determined the EGFR binding epitope of mAb 806. Analysis of mAb 806 binding to EGFR fragments expressed either on the surface of yeast or in an immunoblot format identified a disulfide-bonded loop (amino acids 287–302) that contains the mAb 806 epitope. Indeed, mAb 806 binds with apparent high affinity (~30 nM) to a synthetic EGFR peptide corresponding to these amino acids. Analysis of EGFR structures indicates that the epitope is fully exposed only in the transitional form of the receptor that occurs because EGFR changes from the inactive tethered conformation to a ligand-bound active form. It would seem that mAb 806 binds this small proportion of transient receptors, preventing their activation, which in turn generates a strong antitumor effect. Finally, our observations suggest that the generation of antibodies to transitional forms of growth factor receptors may represent a novel way of reducing normal tissue targeting yet retaining antitumor activity.

The epidermal growth factor receptor (EGFR)[1] is a 170-kDa membrane-bound tyrosine kinase that is responsible for directing the proliferation and differentiation of many different cell types (1, 2). Overexpression of the EGFR has been observed in many epithelial tumors; increased EGFR expression levels usually correlate with poor clinical outcome (3–5). Overexpression of the receptor is often caused by amplification of the *EGFR* gene, an event also linked with EGFR mutation (6, 7). The most common oncogenic EGFR mutation is an extracellular truncation of the EGFR known as the de2–7 EGFR (or EGFRvIII), which is frequently expressed in glioma (6, 8, 9). This truncation results in the removal of 267 amino acids from the extracellular domain of the EGFR and the insertion of a novel glycine, which generates a unique junctional peptide near the N terminus of the de2–7 EGFR (6, 8, 9). Although the de2–7 EGFR does not seem to bind any known ligand with high affinity, it does exhibit low levels of constitutive activation and enhances the tumorigenicity of glioma and breast cells when grown as xenografts in nude mice (10–12).

Inhibition of the EGFR is a strategy for the development of new cancer treatments (13). Potential therapeutics include EGFR-specific antibodies (14) and small molecular weight tyrosine kinase inhibitors (15) of the EGFR. A number of antibodies directed against the extracellular domain of the EGFR have now been tested in the clinic, including EMD 55900 (16), ABX-EGF (17), and C225 (cetuximab) (18), all of which have demonstrated some antitumor activity in patients. The most clinically advanced of these is C225, which is currently being tested in phase II/III clinical trials for the treatment of head and neck, colorectal, and non-small cell lung carcinomas and has been recently approved for use in Europe (19). It has been presumed that the antitumor activity of these antibodies is primarily related to their ability to block ligand binding, but other antitumor mechanisms, such as immune effector function, receptor down-regulation, induction of inappropriate signaling, and interference with receptor dimerization and/or oligomerization could also play a role. One therapeutic limitation of antibody targeting of the wild-type (wt) EGFR is that significant uptake is observed in normal tissue, such as the liver and

* This work was supported in part by National Institutes of Health Grant CA96504, the Ludwig Trust, and the National Health and Medical Research Council of Australia. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors contributed equally to this work.

¶ To whom correspondence should be addressed: Oncogenic Signalling Laboratory, Ludwig Institute for Cancer Research, Austin Hospital, Level 6, Harold Stokes Building, Studley Road, Heidelberg 3084, Australia. Tel.: 613-9496-3068; Fax: 613-9496-5892; E-mail: terry.johns@ludwig.edu.au.

[1] The abbreviations used are: EGFR, epidermal growth factor receptor; wt, wild type; mAb, monoclonal antibody; GH, growth hormone; FACS, fluorescence-activated cell sorting; ELISA, enzyme-linked immunosorbent assay; Bis-Tris, bis(2-hydroxyethyl)iminotris-(hydroxymethyl)methane.

skin (20, 21). At present, targeting of the normal EGFR seems to cause manageable side effects, such as skin rash; however, if these anti-EGFR antibodies were coupled to cytotoxic agents or radioisotopes, significant liver damage would be expected.

The mAb 806 was raised against mouse fibroblast cells expressing the de2–7 EGFR and does not bind to normal tissue expressing the wt EGFR, making it an attractive candidate for cancer therapy (22). When used therapeutically, mAb 806 demonstrated significant antitumor activity against human xenografts expressing either the de2–7 or amplified EGFR (23, 24). Unlike previously described de2–7 EGFR-specific antibodies, which were all raised against peptides and are therefore specific to the unique de2–7 EGFR junctional peptide (25–27), mAb 806 recognizes a different and unknown epitope (28). Although mAb 806 recognizes a large fraction of the de2–7 EGFR, it also binds some of the wt EGFR in tumor cells that overexpress the receptor (28). Scatchard analysis has revealed that the mAb 806 binds ~50% of the de2–7 EGFR recognized by mAb DH8.3, an antibody specific for the de2–7 EGFR junctional peptide (28). In contrast, mAb 806 bound <10% of the wt EGFR overexpressed on A431 tumor cells compared with the wt EGFR-specific mAb 528 (28). It is interesting that mAb 806 can robustly bind to wt EGFR after surface coating on polystyrene or denaturation by SDS-PAGE, suggesting that the antibody recognizes an altered form of the receptor (28). Determination of the mAb 806 binding epitope is important for understanding its mechanism of action, as well as providing a general strategy for developing other tumor-associated antibodies. Using two independent approaches, we have identified the epitope recognized by mAb 806. Taking advantage of recently published crystal structures of the EGFR (29–31), we were also able to assess the unique specificity of mAb 806 and suggest how it might mediate its antitumor activity

EXPERIMENTAL PROCEDURES

Antibodies—The IgG2b mAb 806 and IgG2a mAb 528 specific for the EGFR were produced and purified in the Biological Production Facility (Ludwig Institute for Cancer Research, Melbourne) as described previously (28, 32).

Expression Vectors—The expression vectors pEE14/sEGFR501 and pEE14/sEGFR513, described previously (33), encode the signal peptide and first 501 and 513 amino acids, respectively, of the EGFR ectodomain followed by a c-myc epitope tag, transcribed under the control of the human cytomegalovirus immediate early promoter. The expression vector pEE14/sEGFR310–501 contains cDNA encoding the signal peptide of the EGFR fused in-frame to amino acid residues 310–501 of the ectodomain, terminating with the epitope tag.

A series of overlapping EGFR c-myc-tagged ectodomain fragments, starting at residues 274, 282, 290, and 298 and all terminating at amino acid 501, were generated by PCR. After sequence analysis, the fragments were cloned in-frame into the 3′ end of the human growth hormone (GH) gene expressed from the mammalian expression vector, pSGHVO (34). The nucleotides corresponding to amino acids 287–302 were deleted from the cDNA template encoding residues 274–501 using splice-overlap extension PCR (35) and the resulting fragment sequenced and subcloned into pSGHVO.

Transfections—Human 293T embryonic kidney fibroblasts were maintained in Dulbecco's modified Eagle's medium plus 10% fetal calf serum. The day before transfection, cells were seeded at 8×10^5 per well in six-well tissue culture plates containing 2 ml of media. Cells were transfected with 3–4 μg of plasmid DNA complexed with LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. 24–48 h after transfection, cell cultures were aspirated, and cell monolayers were lysed in 250 μl of lysis buffer (1% Triton X-100, 10% glycerol, 150 mM NaCl, 50 mM HEPES, pH 7.4, 1 mM EGTA, and complete protease inhibitor mixture (Roche Molecular Biochemicals)).

The CR1-loop (dimerization arm) deletion was generated by removing the nucleotides corresponding to amino acids 244–259 and replacing them with a single alanine residue as described previously (29). 293T cells were transfected with this construct, and stable transfectants were selected in the presence of geneticin.

Western Blotting—Aliquots of cell lysate (10–15 μl) were mixed with SDS sample buffer containing 1.5% β-mercaptoethanol, denatured by heating for 5 min at 100 °C, and electrophoresed on 10% NuPAGE Bis-Tris polyacrylamide gels (Invitrogen). Samples were then electrotransferred to nitrocellulose membranes that were rinsed in TBST buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 0.1% Tween 20) and blocked in TBST containing 2.5% skim milk for 30 min at room temperature. Membranes were incubated overnight at 4 °C with 0.5 μg/ml mAb 806 in blocking buffer. Parallel membranes were probed overnight with mAb 9B11 (1:5000; Cell Signaling Technology) to detect the c-myc epitope. Membranes were washed in TBST and incubated in blocking buffer containing horseradish peroxidase-conjugated rabbit anti-mouse IgG (Bio-Rad) at a 1:5000 dilution for 2 h at room temperature. Blots were then washed in TBST and developed using autoradiographic film after incubation with West Pico chemiluminescent substrate (Pierce). For peptide competition experiments, blots were probed for 1 h at room temperature with mAb 806 in the presence of a 100-fold molar excess of competing peptide. After chemiluminescent detection, blots were reprobed with 9B11.

Yeast Surface Display of EGFR Fragments—The pCT yeast display plasmids, modified to contain the appropriate genes encoding for the EGFR fragments, were transformed into the yeast strain EBY100 (36) by electroporation (37) using a Bio-Rad gene pulser transfection apparatus. The plasmid is derived from pRS314 and contains the TRP1 marker that can be used to select for yeast that have incorporated the DNA into their genome. Expression of EGFR proteins on the yeast cell surface was performed as described previously (44). In brief, transformed colonies were grown at 30 °C in minimal media containing yeast nitrogen base, casein hydrolysate, dextrose, and phosphate buffer, pH 7.4, on a shaking platform for approximately 1 day until an A_{600} of 5–6 was reached. Yeast cells were then induced for protein display by transfer to minimal media containing galactose and incubated with shaking at 30 °C for 24 h. Cultures were then stored at 4 °C until analysis.

Antibody Labeling Experiments on the Yeast Cell Surface—Raw ascites fluid containing the c-myc monoclonal antibody 9E10 was obtained from Covance (Richmond, CA). 1×10^6 yeast cells were washed with ice-cold FACS buffer (phosphate-buffered saline containing 1 mg/ml bovine serum albumin) and incubated with either anti-c-myc ascites (1:50 dilution) or human EGFR monoclonal antibody (10 μg/ml) in a final volume of 50 μl for 1 h at 4 °C. The cells were then washed with ice-cold FACS buffer and incubated with phycoerythrin-labeled anti-mouse IgG (1:25 dilution), in a final volume of 50 μl for 1 h at 4 °C, protected from light. After washing the yeast cells with ice-cold FACS buffer, fluorescence data were obtained with a Coulter Epics XL flow cytometer (Beckman Coulter) and analyzed with WinMDI cytometry software (J. Trotter, Scripps University). For determination of linear *versus* conformational epitopes, yeast cells were heated at 80 °C for 30 min, then chilled on ice 20 min before labeling with antibodies.

EGFR-derived Peptides—Peptides (^{287}CGADSYEMEEDGVRKC^{302}, ^{287}CGADSYEMEEDGVRK^{301}, and ^{287}CGADSYEMEEDG^{298}) were synthesized using standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry, purified by reversed-phase high performance liquid chromatography, and characterized by mass spectral analysis. Cyclized peptide was prepared by the overnight aerial oxidation of a dilute peptide solution in alkaline conditions. Linear (reduced) peptide was prepared by dissolving the synthesized peptide in aqueous 10 mM HCl. A sample of the 287–302 peptide was reacted with cyanogen bromide in 70% formic acid under anaerobic conditions to generate fragments corresponding to the N- and C-terminal peptides. The peptides were separated by high performance liquid chromatography on a C18 Vydac column using an acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. The authenticity of the peptides was subsequently characterized by mass spectrometry and N-terminal sequencing. A sample of *S*-carboxymethylated peptide was produced by reacting the peptide with dithiothreitol in 0.5 M sodium bicarbonate, pH 8.6, followed by the addition of iodoacetamide. The *S*-carboxymethylated peptide was subsequently purified by reverse phase-high performance liquid chromatography as described above.

ELISA—The wells of white polystyrene 96-well plates (Greiner Lumitrac 600) were coated with 2 μg/ml 501-Fc, a variant form of sEGFR501 fused to the human Fc constant region,[2] in 10 mM sodium citrate, pH 5.9, and then blocked with 0.5% chicken ovalbumin in TBST. After washing with TBST, solutions (100 μl/well) of 0.5 μg/ml mAb 806 and varying concentrations of peptides were added to the wells. Plate-

[2] T. E. Adams, E. Koziolek, P. A. Hoyne, E. De Silva, G. O. Lovrecz, N. M. McKern, A. W. Burgess, and C. W. Ward, unpublished results.

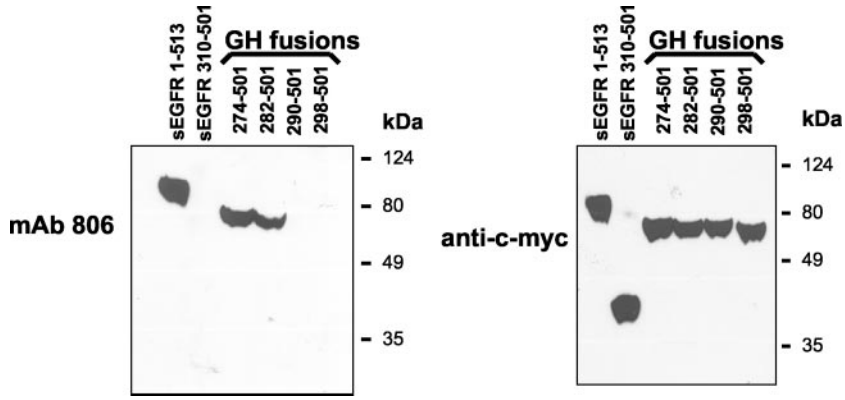

FIG. 1. **Reactivity of mAb 806 with fragments of the EGFR.** Lysates from 293T cells transfected with vectors expressing soluble fragments of the EGFR (1–513 and 310–501) or GH/EGFR fragment fusion proteins (GH-(274–501), GH-(282–501), GH-(290–501), and GH-(298–501)) were resolved by SDS-PAGE, transferred to membrane, and immunoblotted with mAb 806 (*left*) or the anti-myc antibody 9B11 (*right*).

bound mAb 806 was detected using goat anti-mouse IgG-HRP (Bio-Rad) and West Pico chemiluminescent substrate (Pierce) and quantitated using a Wallac Victor 1420 counter (PerkinElmer Life and Analytical Sciences). In some assays, mAb 806 binding was analyzed using 96-well plates coated with the sEGFR501 as described previously (28).

Surface Plasmon Resonance (BIAcore)—A BIAcore 3000 was used for all experiments. The peptides containing the putative mAb 806 epitope were immobilized on a CM5 sensor chip using amine or thiol-disulfide exchange coupling at a flow rate of 5 μl/min (38). The 806 antibody was passed over the sensor surface at a flow rate of 5 μl/min at 25 °C. The surfaces were regenerated between runs by injecting 10 mM HCl at a flow rate of 10 μl/min.

Flow Cytometry Analysis of EGFR-transfected Cells—Cultured 293T cells transfected with different EGFR constructs were analyzed for cell surface EGFR expression using mAb 528 or mAb 806. 1×10^6 cells were incubated with 5 μg/ml primary antibody, in phosphate-buffered saline containing 1% human serum albumin for 30 min at 4 °C. After washing with cold phosphate-buffered saline, 1% human serum albumin, cells were incubated for an additional 30 min with fluorescein isothiocyanate-coupled goat anti-mouse antibody at 4 °C (1:100 dilution; Calbiochem). Fluorescence data were obtained using an Epics Elite ESP flow cytometer (Beckman Coulter). A minimum of 5000 cell events was observed and analyzed using EXPO software for Windows (version 2).

RESULTS

Identification of the mAb 806 Epitope by Immunoblotting of EGFR Fragments—To determine the broad location of the mAb 806 epitope, 293T cells were transfected with expression vectors encoding a number of EGFR ectodomain isoforms. Cell lysates containing 1–513 and 310–501 c-myc-tagged EGFR fragments were resolved by SDS-PAGE and immunoblotted with mAb 806. Although mAb 806 showed strong reactivity to the 1–513 fragment, it did not bind at all to the 310–501 fragment of the EGFR (Fig. 1, *left*). The presence of the 310–501 fragment on the membrane could be detected using mAb 9B11, which is specific for the c-myc tag (Fig. 1, *right*). In other experiments, we established that mAb 806 also bound the soluble EGFR501 fragment (33) in Western blots (data not shown). Given that mAb 806 binds the de2–7 EGFR (28), which has amino acids 6–273 deleted, we concluded that the mAb 806 epitope must be contained within residues 274–310. To delineate the epitope of mAb 806, we expressed a series of c-myc-tagged EGFR fragments, all terminating at amino acid 501, fused to the carboxyl terminus of human GH. The mAb 806 reacted with both the 274–501 and 282–501 EGFR fragments but failed to bind to segments commencing at amino acid 290 or 298 (Fig. 1, *left*). The presence of all GH-EGFR fusion proteins was confirmed using the c-myc antibody (Fig. 1, *right*). Thus, the mAb 806 epitope must be contained within amino acids 282–310. Furthermore, although the epitope could extend beyond amino acid 290, the 282–290 region must contain some of the amino acid residues critical for mAb 806 reactivity in this particular immunoblotting assay.

Identification of the mAb 806 Epitope by Yeast Surface Display of EGFR Fragments—We used a second approach to determine the location of the mAb 806 epitope. Fragments encompassing extracellular domains of the EGFR were expressed on the surface of yeast and tested for mAb 806 binding by indirect immunofluorescence using flow cytometry. The mAb 806 recognized both the yeast-displayed EGFR fragments 1–501 and 1–621 (Fig. 2*A*). In addition, the mAb 806 bound the EGFR fragment 273–621, which corresponds to the extracellular domain of the de2–7 EGFR (Fig. 2*A*). The mAb 806 could not recognize the EGFR fragments 294–543 or 475–621 (Fig. 2*A*), demonstrating that at least part of the mAb 806 epitope must be contained within the region between amino acids 274 and 294 (see amino acids 282–290, identified above). Given that these two different approaches identified the same region as critical for mAb 806 binding, we were confident that this region of the EGFR must contain an energetically important portion of the mAb 806 epitope. It was interesting that heat denaturation at 80 °C of the EGFR fragment 1–501 had no effect on mAb 806 binding, suggesting that the epitope is not conformational or discontinuous (Fig. 2*B*). This result is consistent with our previous data, which demonstrated that mAb 806 recognizes a large proportion of wt EGFR once the receptor is denatured by SDS-PAGE (28).

Binding of mAb 806 to an EGFR-derived Peptide Containing the Putative Epitope—A comparison of the amino acid sequences of the human and mouse EGFR spanning residues 274–310 revealed that of a total of five amino acid differences, four resided within the region bounded by Cys-287 to Cys-302. This is important because mAb 806 does not bind the denatured murine EGFR (data not shown). The deletion of the nucleotide sequence encompassing this cysteine loop from the expression construct encoding GH fused to amino acids 274–501 of the EGFR (GH-(274–501Δ287–302)), completely eliminated immunoreactivity with mAb 806 after immunoblotting of transfected cell lysates (Fig. 3*A*, *left*), whereas recognition by the myc tag-specific antibody was unaffected (Fig. 3*A*, *right*). A peptide (^{287}CGADSYEMEEDGVRKC^{302}) proposed to contain the putative mAb 806 epitope was synthesized. This peptide inhibits the binding of mAb 806 to EGFR fragments 1–501 and 274–501 in an immunoblot (Fig. 3*B*, *top*). The presence of the EGFR fragments on both portions of the immunoblot was confirmed by re-probing with anti-c-myc (Fig. 3*B*, *bottom*). The 287–302 EGFR peptide in solution was also able to inhibit the binding of mAb 806 to the immobilized 1–501 fragment as assayed by ELISA (Fig. 3*C*). It was interesting that a shorter peptide (amino acids 287–298) did not inhibit the binding of mAb 806 at the concentrations tested (Fig. 3*C*). Thus, the mAb 806 epitope seems to be contained within residues 287–302, which form a disulfide-constrained loop in the EGFR

We also tested the ability of the 287–302 EGFR peptide to inhibit the binding of mAb 806 to immobilized 501-Fc, a soluble

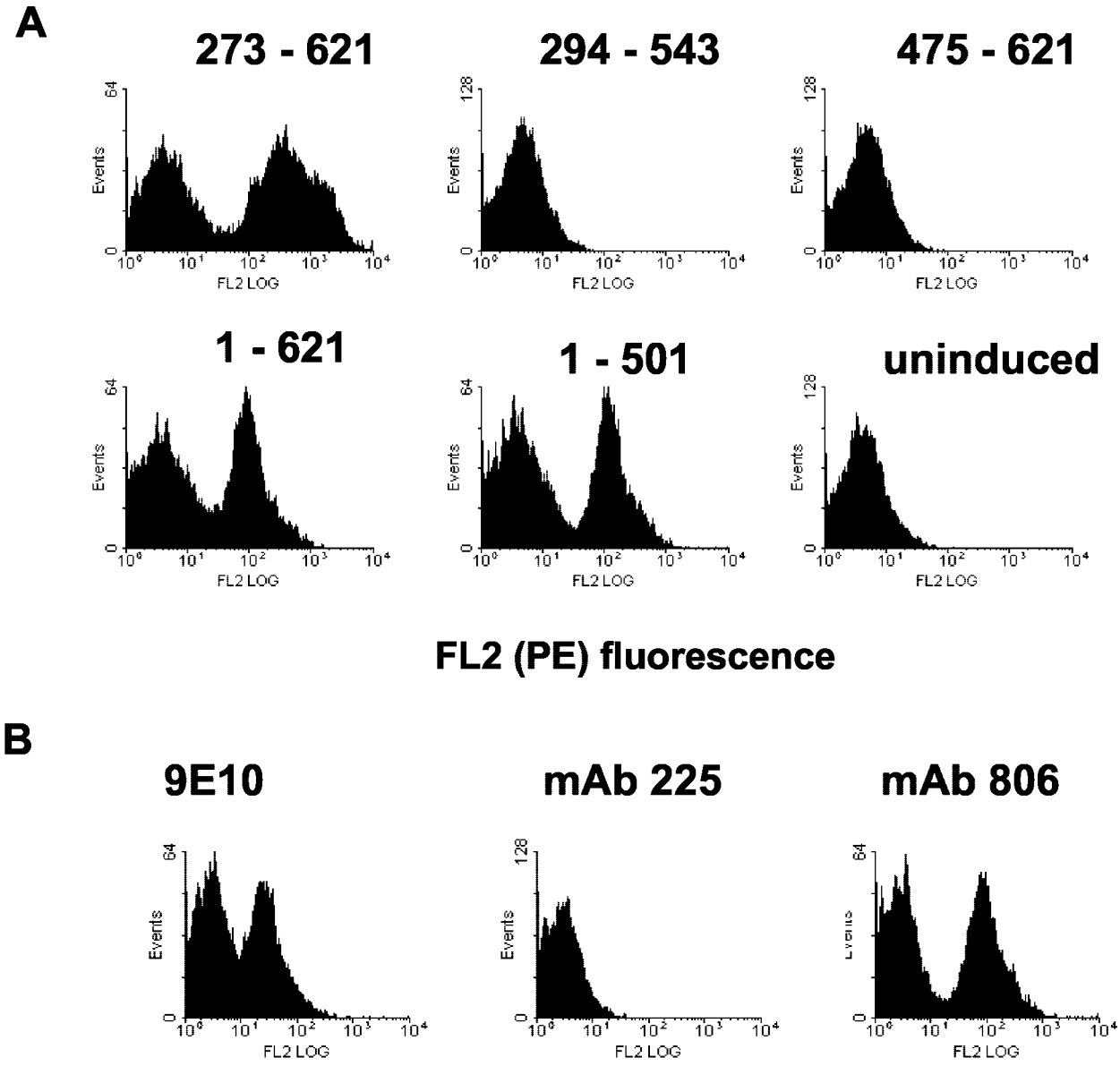

FIG. 2. **Reactivity of mAb 806 with fragments of the EGFR displayed on yeast.** *A*, representative flow cytometry histograms depicting the mean fluorescence signal of mAb 806 labeling of yeast-displayed EGFR fragments. With yeast display, a percentage of cells do not express protein on their surfaces, resulting in two histogram peaks. mAb 806 did not bind to the uninduced negative control. *B*, the 1–501 EGFR fragment was denatured by heating yeast pellets to 80 °C for 30 min. The linear C-terminal c-myc tag on the 1–501 fragment was still recognized by the 9E10 antibody, demonstrating that heat treatment does not compromise the yeast surface displayed protein. The conformation-sensitive mAb 225 was used to confirm denaturation.

dimeric version of the 1–501 EGFR fragment fused to the Fc region of human IgG1. Oxidized, reduced, and aged (*i.e.* moderately aggregated) peptide all inhibited binding of mAb 806 to the 501-Fc in a dose-dependent manner (Fig. 4*A*). A peptide containing reduced and *S*-carboxymethylated cysteine residues was unable to inhibit the binding of mAb 806, indicating that one or both cysteine residues contribute to the mAb 806 epitope (Fig. 4*B*). N-terminal (CGADSYEM) or C-terminal (EEGVRKC) peptide fragments generated by cyanogen bromide cleavage were incapable of inhibiting mAb 806 binding (Fig. 4*B*), implying that the epitope spans the internal methionine residue. These data provide further confirmation that the mAb 806 epitope is contained within the EGFR-derived peptide 287–302.

The 287–302 EGFR peptide was coupled to a CM5 sensor chip by thiol-disulfide exchange coupling at a terminal cysteine residue, and mAb 806 binding was analyzed by surface plasmon resonance (BIAcore). The mAb 806 bound to the immobilized peptide in a dose-dependent manner (Fig. 5*A*) with an apparent affinity of 30 nM (Fig. 5*A*), which is consistent with the affinity for the de2–7 EGFR obtained using Scatchard analysis on live cells (28). mAb 806 binding to a blank channel, a cysteine-blocked channel, or an irrelevant peptide were all less than 1% of the binding to the 287–302 EGFR peptide (data not shown). Because the affinity of mAb 806 for this peptide is similar to the affinity displayed for de2–7 EGFR, the peptide seems to contain all the major determinants that contribute to the epitope. Because the peptide was immobilized using thiol coupling and cannot form an intramolecular disulfide bond, this observation further demonstrates that the peptide does not have to be covalently cyclized for high affinity mAb 806 binding. However, we cannot exclude the possibility that some of the peptide is in a loop structure as a result of the immobilization of both cysteines. We also immobilized the 287–302 EGFR peptide via amine coupling and showed that mAb 806 still bound (Fig. 5*B*)

We then tested the ability of several soluble EGFR-derived

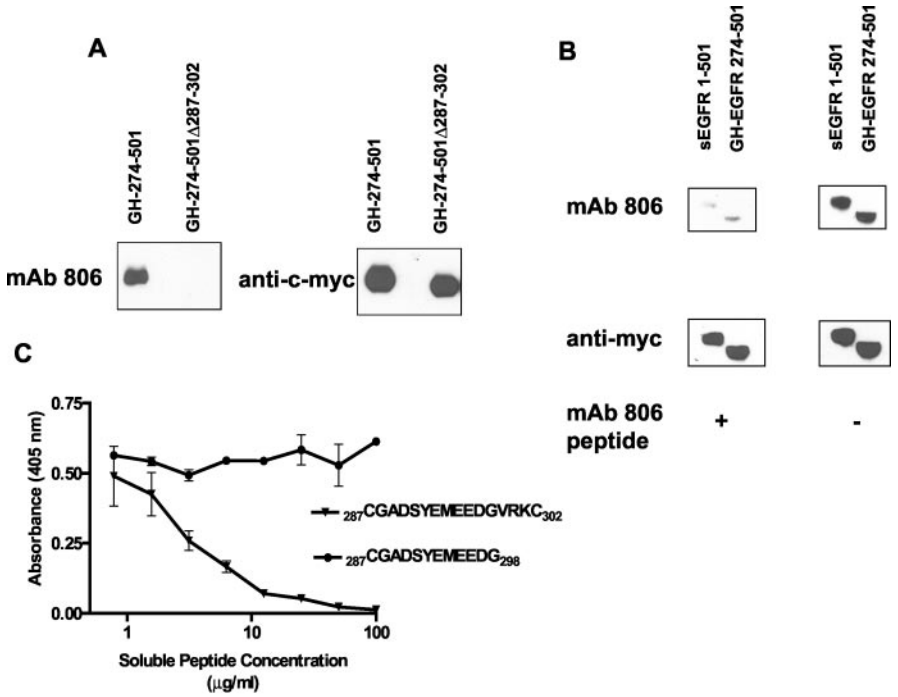

FIG. 3. **Inhibition of mAb 806 binding with an EGFR-derived peptide.** *A*, lysates from 293T cells transfected with vectors encoding GH-EGFR fusion proteins GH-(274–501) and GH-(274–501Δ287–302) were resolved by SDS-PAGE, transferred to membrane, and probed as in Fig. 1 with mAb 806 (*left*) or anti-c-myc (*right*). *B*, the EGFR fragments 1–501 and GH-(274–501) were immunoblotted with mAb 806 (*top*) as described in Fig. 1 in the presence or absence of the 287–302 EGFR peptide. Presence of EGFR fragments on the *left blot* was confirmed after mAb 806 immunoblotting by reprobing membranes with anti-myc mAb 9B11 (*bottom*). *C*, ELISA plates were coated with the soluble 1–501 EGFR fragment and then incubated with mAb 806 in the presence of increasing concentrations of the 287–302 or 287–298 EGFR peptides. Data are expressed as mean A_{405} ± S.D.

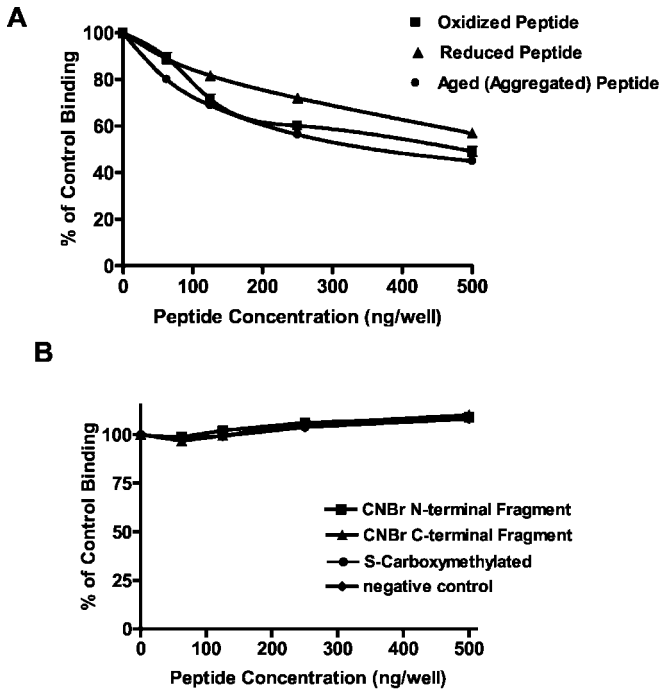

FIG. 4. **Inhibition of mAb 806 binding with chemically modified 287–302 EGFR peptide.** *A*, ELISA plates were coated with 501-Fc and then incubated with mAb 806 in the presence of increasing concentrations of oxidized, reduced, and aged 287–302 EGFR peptide (prepared as described under "Experimental Procedures"). Data are expressed as mean percentage of control binding ± S.D. (error bars are too small to be visible). *B*, ELISA plates were coated with 501-Fc and then incubated with mAb 806 in the presence of increasing concentrations of *S*-carboxymethylated 287–302 EGFR peptide or the N-terminal (CGADSYEM) and C-terminal (EEGVRKC) peptides generated from the CNBr cleavage of the 287–302 EGFR peptide. Data are expressed as mean percentage of control binding ± S.D. (error bars are too small to be visible).

peptides to block the binding of mAb 806 to amine-immobilized 287–302 EGFR peptide. As expected, the soluble 287–302 EGFR peptide inhibited mAb 806 binding in a dose-dependent manner (Fig. 5*B*, *top*). Consistent with our ELISA data (Fig. 3*B*) the 287–298 EGFR peptide was unable to prevent binding of mAb 806 even when used in vast excess (Fig. 5*B*, *middle*). An additional peptide simply lacking Cys-302 (*i.e.* amino acids 287–301) was able to weakly inhibit mAb 806 binding in a dose-dependent manner (Fig. 5*B*, *lower*). These observations confirm that the amino acid residue Cys-302 is required for high affinity mAb 806 binding.

Structural Analysis of the mAb 806 Epitope and Its Relationship to EGFR Activation—Several recent crystallographic studies have described the structure of the EGFR extracellular domain in several conformations with bound ligand (29–31). Thus, we assessed the structural location of the mAb 806 epitope to provide insight into its unique receptor specificity. In the structures, residues 287–302 form a disulfide-bonded loop (Fig. 6*A*), located at the C-terminal portion of the cysteine rich CR1-domain (Fig. 6*B*, *maroon*). It is interesting that this region of the EGFR is poorly resolved in all the published EGFR structures, suggesting a relative degree of protein flexibility in this region. A considerable portion of the 287–302 EGFR loop is buried within the EGFR; however, there are two regions of the epitope that are more exposed and are potentially accessible by antibody. The first region is centered on Asp-290 (Fig. 6*C*, highlighted in *maroon* in the *left-side view*) and the second of these is focused on residue Asp-297, which can be observed when the molecule is rotated 180° (Fig. 6*C*, highlighted in *maroon* in the *right-side view*).

The tethered form of the EGFR depicted in Fig. 6*C* is an inactive conformation of the receptor. In this state, the EGFR CR2-domain interacts with the CR1-domain in a manner that prevents the dimerization arm, a small loop contained within the CR1-domain, from interacting with the dimerization arm of other EGFR molecules. Untethering of the EGFR leads to an extended form of the receptor in which the dimerization arm is exposed (Fig. 6*D*, *left*), allowing the receptor dimerization to occur (Fig. 6*B*). Assuming free equilibration between tethered and untethered configurations, it has been suggested that nearly 95% of the EGFR on the cell surface would be in the tethered conformation (39). The remaining EGFR would be in the active dimer or extended untethered conformation. Addi-

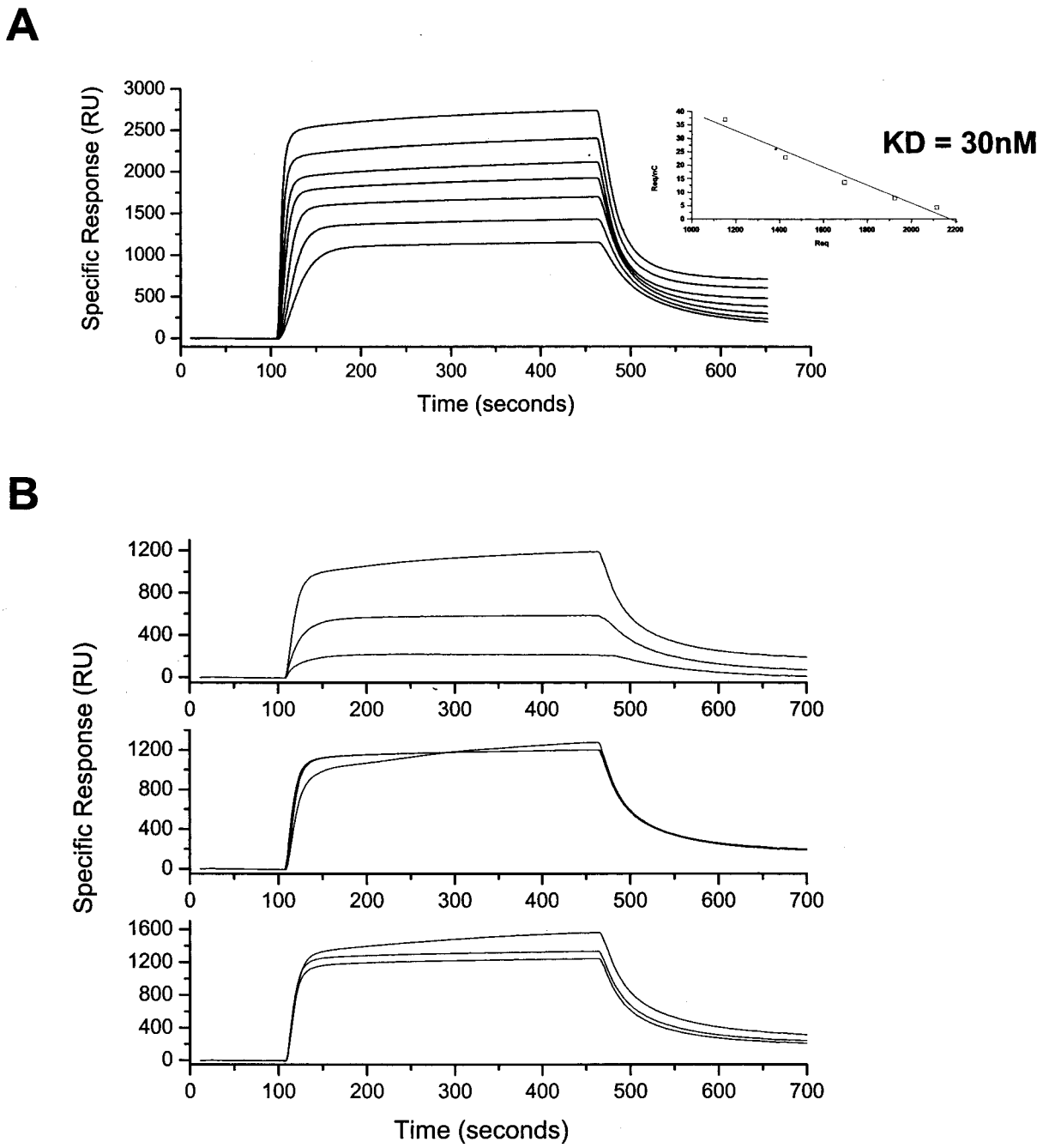

FIG. 5. **Analysis of mAb 806 binding to peptides by BIAcore.** *A*, the 287–302 EGFR peptide was immobilized on the surface by thiol coupling and the mAb 806 antibody passed over at increasing concentrations (31.25, 62.5, 125, 250, 500, 1000, and 2000 nM). Binding affinity was then determined by Scatchard analysis of the equilibrium binding data (*inset*). *B*, the 287–302 EGFR peptide was immobilized on the surface by amine coupling, and the mAb 806 antibody at a concentration of 500 nM was passed over the sensor surface in the presence of the soluble EGFR peptides 287–302 (*upper*), 287–298 (*middle*), or 287–301 (*lower*) EGFR peptides (5 and 10 μM).

tion of ligand drives the receptor into the active dimeric form (Fig. 6*B*).

With respect to possible mAb 806 binding sites, the only residues accessible in the tethered form of the receptor are those centered on Asp-297. However, given that mAb 806 binds to only 5–10% of the EGFR in cell lines overexpressing the receptor, it is extremely unlikely that mAb 806 binds to the tethered form of the EGFR, which probably forms 95% of EGFR on the cell surface. Based on the structural information presented in Fig. 6, dimerization of the EGFR does not expose any additional amino acid residues within the mAb 806 epitope and therefore would not be a target for mAb 806 binding. Given the large size of an antibody, none of the exposed amino acids centered on Asp-290 would probably be accessible to the mAb 806 in either the tethered or active dimeric conformations. As the EGFR moves from the tethered conformation to the active dimeric state, it must pass through a transitional extended state. This transitional untethered form of the EGFR (Fig. 6*D*) may be monomeric, or possibly an inactive dimer, and would be comparatively rare on the cell surface consistent with the level of mAb m806 binding. It is significant that in this transitional form of the receptor, the residues around Asp-290, as well as a number of amino acids normally buried (*e.g.* Tyr-292 and Met-294), would be accessible to antibody binding. Spatial consid-

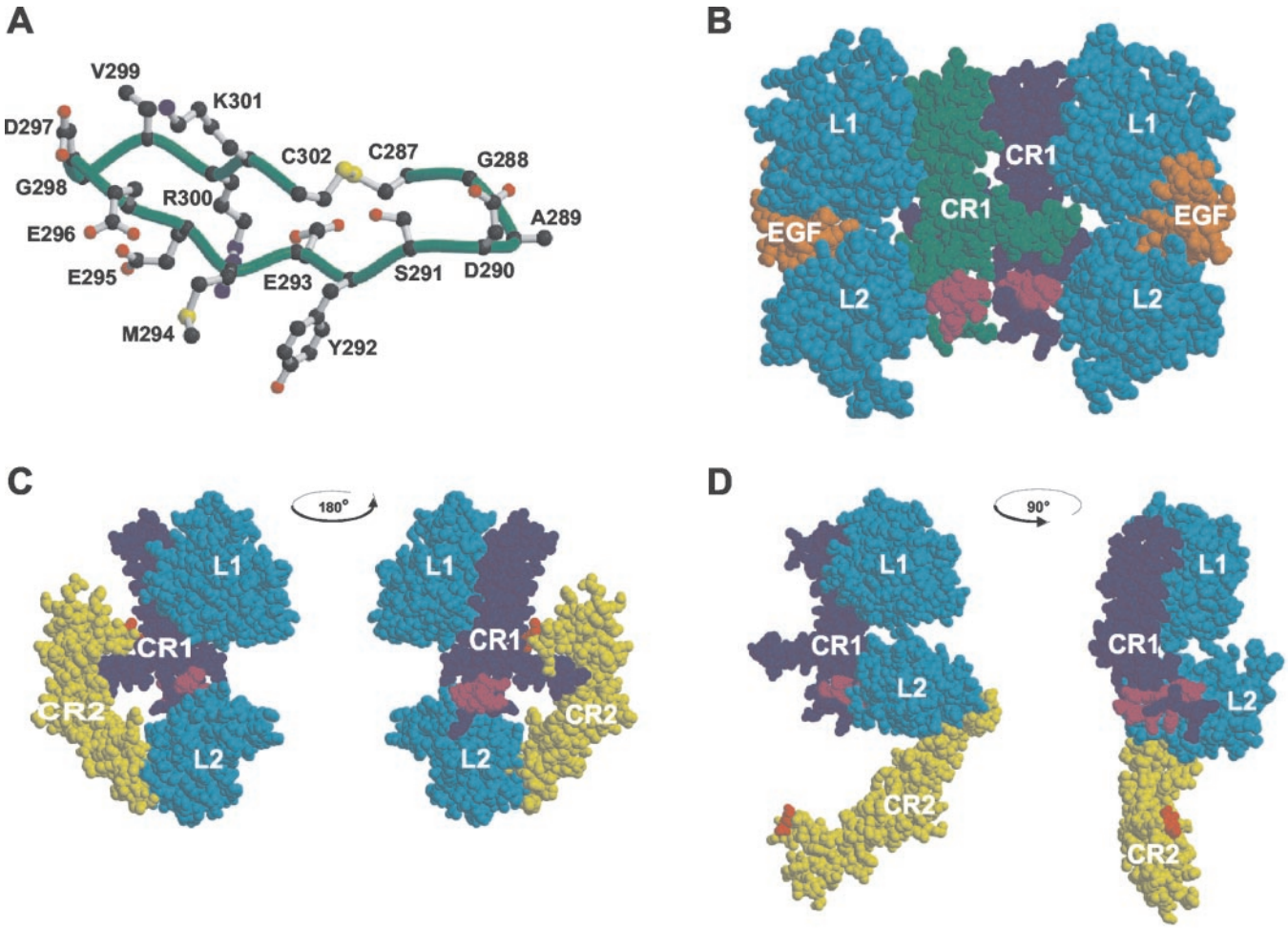

FIG. 6. **Location of the mAb 806 epitope within the EGFR structure.** *A*, carbon trace showing the structure of the disulfide-bonded loop containing the mAb 806 epitope. *B*, space-filled model of the ligand-bound dimeric form of the EGFR. The dimer is predominantly stabilized by the two dimerization arms located in the CR1-loop of each EGFR molecule. *C*, tethered form of the EGFR showing the autoinhibitory interaction between domains CR1 and CR2, which prevents dimerization. *D*, extended (transitional) form of the EGFR clearly showing the dimerization arm (*left*) of the CR1-loop poised and ready for interaction with a second loop on an adjacent molecule. EGF ligand is shown in *orange*, glycosylation site at amino acid 579 in *red*, and mAb 806 epitope in *maroon*. EGFR structures (29–31) and a possible receptor activation mechanism (29–31, 38) have been described in detail previously.

erations strongly indicate that binding of mAb 806 to the region near Asp-290 may require interaction with amino acids outside the disulfide-bonded 297–302 loop, which is inconsistent with our affinity data suggesting that the entire mAb 806 epitope is contained within this loop. If we eliminate Asp-290 as a binding region, then mAb 806 must interact with the region around Tyr-292/Met-294 although the epitope may extend further to include Asp-297. Taken together, the only consistent conclusion is that mAb 806 binds to amino acids transiently exposed in the extended form of the EGFR before it undergoes ligand-mediated active dimerization.

Binding of mAb 806 to Constitutively Untethered Forms of the EGFR—To confirm that mAb 806 preferentially binds the untethered EGFR, we stably expressed a mutation of the EGFR lacking the CR1 dimerization arm (deCR1-loop) (29) in 293T cells. This region was chosen for its role in forming active EGFR dimers and because the CR1 dimerization arm is also integrally involved in the CR1-CR2 interactions associated with tethering. Thus, the deCR1, like the de2–7 EGFR, should be constitutively untethered. The parental 293T cells express a low level of wt EGFR ($\sim 1 \times 10^4$ EGFR/cell), as evidenced by the binding of mAb 528 by FACS (Fig. 7). As expected, mAb 806 does not bind the endogenous EGFR expressed in these cells. The de2–7 EGFR, like the deCR1-loop, should not be

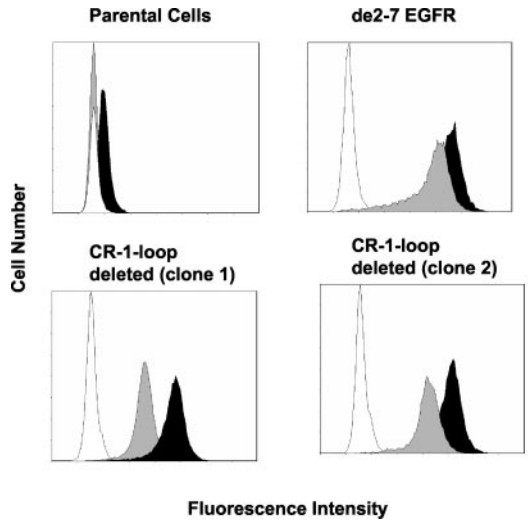

FIG. 7. **Flow cytometric analysis of 293T cells expressing CR1-loop deletions of the EGFR.** Parental 293 cells, which express low amounts of endogenous wt EGFR, were transfected with the de2–7 EGFR or the deCR1-loop EGFR (two independent clones) and labeled with either an irrelevant IgG2b antibody (*open histograms*), mAb 528 (*black histograms*), or mAb 806 (*gray histograms*).

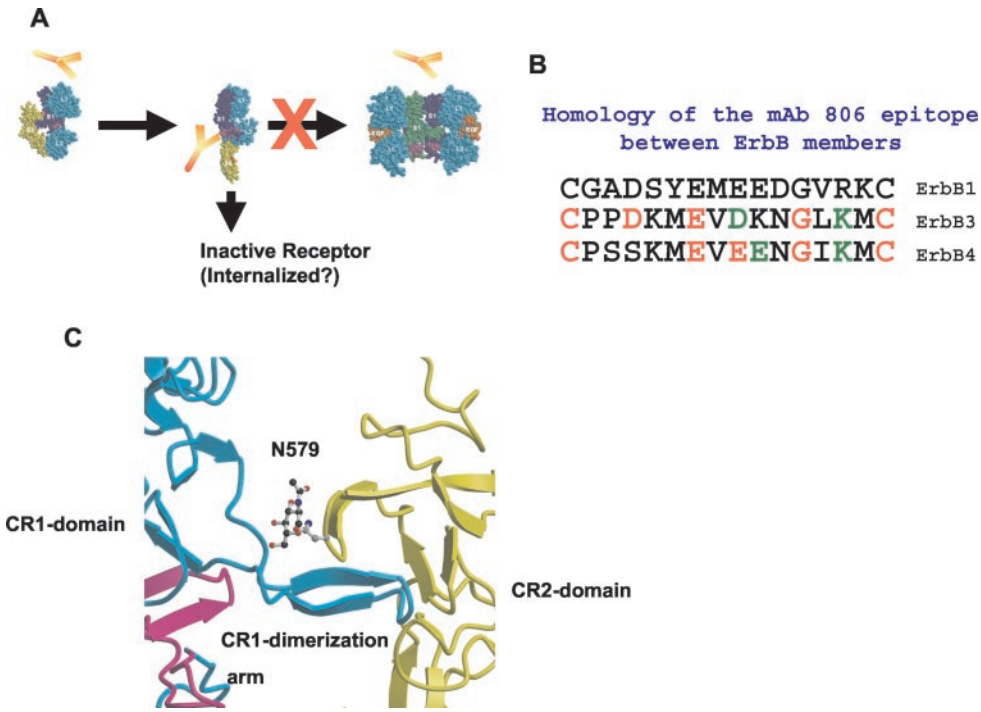

FIG. 8. *A*, possible antitumor mechanism of mAb 806. The mAb 806 cannot bind the inactive EGFR; as the receptor untethers, however, the mAb 806 epitope becomes exposed, allowing antibody binding. Binding of mAb 806 to the receptor would almost certainly prevent active dimerization, and hence EGFR signaling, and may induce EGFR internalization. *B*, homology of the mAb 806 epitope in ErbB3 and ErbB4. Amino acids conserved in ErbB1 are shown in *red*, and residues displaying conservation of charge are shown in *green*. *C*, the CR1-CR2 dimer interface. The first carbohydrate moiety attached to Asn-579 is clearly visible in the crystal structure and is located at the CR1-CR2 dimer interface. In cells overexpressing the EGFR, this site is glycosylated only 80% of the time. Differences in glycosylation may effect the dynamics of tethering and hence mAb 806 reactivity.

able to tether. Transfection of 293T cells with the de2–7 EGFR led to robust binding of mAb 806, as shown previously in other cell lines (Fig. 7). Binding of the highly conformation-dependent mAb 528 to the deCR1-loop EGFR confirms that there is no gross change to its conformation (Fig. 7). Indeed, we have shown previously that the deCR1-loop EGFR can bind ligand, further supporting the notion that its overall structure remains intact (29). Flow cytometry analysis of two independent deCR1-loop EGFR-expressing clones clearly showed binding of mAb 806 (Fig. 7). Thus, consistent with our hypothesis, mAb 806 seems to bind the untethered EGFR before it forms an active dimer. mAb 806 also shows increased binding to EGFR point mutants that have a reduced capacity to tether.[3]

DISCUSSION

The mAb 806 was generated after immunization with mouse fibroblasts expressing the de2–7 EGFR and was selected by mixed hemadsorption assay for high reactivity to de2–7 EGFR and negligible activity against the wt EGFR (22). Further characterization revealed that mAb 806 could recognize cell lines and glioma specimens when the wt EGFR was overexpressed, especially when the *EGFR* gene was amplified, but not normal tissue (22). To elucidate the mechanism underpinning the unique specificity of mAb 806, we sought to determine its epitope. Using two independent techniques, we identified a disulfide-bonded loop (amino acids 287–302) that contains the mAb 806 epitope. Because the mAb 806 affinity to a synthetic peptide encompassing residues 287–302 is similar to what we have previously measured by Scatchard analysis with de2–7 EGFR-expressing cell lines (28), we are confident it contains the complete epitope sequence. It is clear that the peptide does not have to be constrained in a disulfide-bonded loop for antibody binding because the mAb 806 recognized reduced peptide in solution, thiol-immobilized peptide, and weakly bound a soluble peptide with Cys-302 deleted.

Both immunoprecipitation and Scatchard analysis demonstrated that mAb 806 recognizes between 5 and 10% of the wt EGFR expressed on the surface of A431 tumor cells (28), a cell line overexpressing the receptor because of an amplification of the *EGFR* gene. Despite binding to a low proportion of receptors, mAb 806 displays robust antitumor activity against A431 xenografts grown in nude mice (23, 24). Our observation that mAb 806 preferentially binds the untethered EGFR suggests a probable mechanism for this antitumor activity. As an EGFR molecule untethers, it enters a transitional state between inactive tether and active dimer (39). This transitional untethered form of the EGFR is engaged by mAb 806. Binding of mAb 806 then prevents the formation of signaling-capable EGFR dimers (Fig. 8). Thus, although mAb 806 binds only a low percentage of the EGFR at any given time, over an extended period of time it would be capable of inhibiting a substantial proportion of EGFR signaling, which in turn generates an antitumor effect *in vivo*. The fate of mAb 806-bound wt EGFR is unknown, although we have previously shown that the mAb 806-de2–7 EGFR complex is internalized (28). On the other hand, the mAb 806-wt EGFR could remain trapped on the surface in an inactive form, as is the case after treatment of cells with low molecular weight tyrosine kinase inhibitors specific to the EGFR (32, 40). In contrast to the low reactivity seen with the wt EGFR, mAb 806 recognizes (compared with DH8.3, an antibody specific for the mutant receptor) approximately

[3] Walker, F., Orchard, S. G., Jorissen, R. N., Hall, N. E., Zhang, H. H., Hoyne, P. A., Adams, T. E., Johns, T. G., Ward, C., Garrett, T. P. J., Zhu, H. J., Nerrie, M., Scott, A. M., Nice, E. C., and Burgess, A. W. (2004) *J. Biol. Chem.* **279,** 22387–22398.

one-half of the de2–7 EGFR molecules expressed on a tumor cell surface (28). The increased reactivity of mAb 806 for the de2–7 EGFR is consistent with the fact that this mutant receptor lacks the CR1 dimerization loop and therefore cannot assume the tethered conformation.

If mAb 806 recognizes a normal but comparatively low abundance transitional conformation of the EGFR, why does it fail to bind normal tissues or cell lines expressing "average" levels of EGFR? This observation seems unrelated to sensitivity of detection, because in a previous study, we showed that iodinated mAb 806 did not bind to a U87MG glioma (1×10^5 EGFR/cell) cell pellet containing 1×10^7 cells (28), which, based on our studies with A431 cell pellet, should have been more than sensitive enough to measure low level binding. Thus, other factors seem to increase the mAb 806 reactivity in cells overexpressing the EGFR. These factors most probably enhance EGFR untethering and could include autocrine ligand production, ligand-independent receptor activation (an event largely restricted to cells that overexpress the receptor), alterations in glycosylation, or a combination of any of these possibilities. Indeed, we have already shown that glycosylation influences mAb 806 reactivity. mAb 806 preferentially recognizes the high mannose form of the EGFR that normally resides within the endoplasmic reticulum,[4] presumably because the epitope is exposed in this nascent form of the receptor, possibly because it is constitutively untethered. In cells overexpressing the EGFR, some of this high mannose receptor is misdirected to the cell surface, where it can be recognized by mAb 806 (41).[4] Recently Zhen *et al.* (42) characterized EGFR glycosylation in A431 cells and showed that the Asn-579 is glycosylated on only 80% of the receptor molecules. It is interesting that this glycosylation site is located in the CR2-domain (shown in *red* in Fig. 6*C*) and would be buried in the interface between the CR1- and CR2-domains when the EGFR is in the tethered conformation (Fig. 8*C*). Thus, the absence of glycosylation at this particular residue could influence the kinetics of untethering and therefore mAb 806 binding, especially in cells that overexpress the receptor. We are currently examining whether glycosylation at Asn-579 influences receptor activation, mAb 806 binding, and EGF binding. In conclusion, we propose that mAb 806 recognizes the untethered EGFR and that untethering is enhanced in cell lines overexpressing the receptor.

Even though the sequence homology of the region equivalent to the mAb 806 epitope is relatively low in ErbB3/B4 family members, the size and location of the disulfide-bonded loop is conserved. Furthermore, two amino acid residues are completely conserved (Glu-293 and Gly-298), and there are two residues in which charge is conserved (Glu-295 and Arg-300). Finally, the overall structure of ErbB3 is very similar to that of the EGFR in that it adopts a tethered conformation that presumably untethers during activation (43). Taken together, this suggests that antibodies targeted to the equivalent cysteine loop in ErbB3/B4 may have properties similar to those of mAb 806 (*i.e.* specificity restricted to tumors and the ability to block receptor activation). More broadly, our data suggest that the generation of antibodies to transitional forms of growth factor receptors may represent a novel way of reducing normal tissue targeting while retaining antisignaling activity. It is clear that choosing epitopes fulfilling this criteria will be difficult, but comparisons between the structure of active (ligand bound) receptors and their inactive counterparts may identify amino acids transiently exposed during receptor conformational changes. Finally, mAb 806 was generated by immunizing mice with cells expressing a constitutively active mutation of the EGFR and selecting for antibodies specific to this mutated receptor. Thus, immunization with constitutively active receptor may provide a generalized strategy that increases the likelihood of identifying antibodies recognizing transitional forms of the receptor.

Acknowledgments—We thank Dr. Dan Leahy (JHMI, Johns Hopkins University) for the pSGHVO expression vector and John Bentley and Kellie Cartledge for their help with some of the experiments.

[4] T. G. Johns, I. Ira Mellman, G. A. Cartwright, G. Ritler, L. J. Old, A. W. Burgess, and A. M. Scott, submitted.

REFERENCES

1. Wells, A. (1999) *Int. J. Biochem. Cell Biol.* **31,** 637–643
2. Olayioye, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000) *EMBO J.* **19,** 3159–3167
3. Mendelsohn, J. (2002) *J. Clin. Oncol.* **20,** (suppl.) 1–13.
4. Arteaga, C. L. (2002) *Semin. Oncol.* **29,** 3–9
5. Nicholson, R. I., Gee, J. M., and Harper, M. E. (2001) *Eur. J. Cancer* **37,** Suppl. 4, 9–15
6. Frederick, L., Wang, X. Y., Eley, G., and James, C. D. (2000) *Cancer Res.* **60,** 1383–1387
7. Malden, L. T., Novak, U., Kaye, A. H., and Burgess, A. W. (1988) *Cancer Res.* **48,** 2711–2714
8. Wong, A. J., Ruppert, J. M., Bigner, S. H., Grzeschik, C. H., Humphrey, P. A., Bigner, D. S., and Vogelstein, B. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 2965–2969
9. Sugawa, N., Ekstrand, A. J., James, C. D., and Collins, V. P. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 8602–8606
10. Wikstrand, C. J., Reist, C. J., Archer, G. E., Zalutsky, M. R., and Bigner, D. D. (1998) *J. Neurovirol.* **4,** 148–158
11. Tang, C. K., Gong, X. Q., Moscatello, D. K., Wong, A. J., and Lippman, M. E. (2000) *Cancer Res.* **60,** 3081–3087
12. Nishikawa, R., Ji, X. D., Harmon, R. C., Lazar, C. S., Gill, G. N., Cavenee, W. K., and Huang, H. J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 7727–7731
13. de Bono, J. S., and Rowinsky, E. K. (2002) *Trends Mol. Med.* **8,** (suppl.) 19–26
14. Herbst, R. S., and Shin, D. M. (2002) *Cancer* **94,** 1593–1611
15. Wakeling, A. E. (2002) *Curr. Opin. Pharmacol.* **2,** 382–387
16. Stragliotto, G., Vega, F., Stasiecki, P., Gropp, P., Poisson, M., and Delattre, J. Y. (1996) *Eur. J. Cancer* **32A,** 636–640
17. Lynch, D. H., and Yang, X. D. (2002) *Semin. Oncol.* **29,** 47–50
18. Herbst, R. S., Kim, E. S., and Harari, P. M. (2001) *Expert. Opin. Biol. Ther.* **1,** 719–732
19. Herbst, R. S., and Langer, C. J. (2002) *Semin. Oncol.* **29,** 27–36
20. Divgi, C. R., Welt, S., Kris, M., Real, F. X., Yeh, S. D., Gralla, R., Merchant, B., Schweighart, S., Unger, M., and Larson, S. M. (1991) *J. Natl. Cancer Inst.* **83,** 97–104
21. Busam, K. J., Capodieci, P., Motzer, R., Kiehn, T., Phelan, D., and Halpern, A. C. (2001) *Br. J. Dermatol.* **144,** 1169–1176
22. Jungbluth, A. A., Stockert, E., Huang, H. J., Collins, V. P., Coplan, K., Iversen, K., Kolb, D., Johns, T. J., Scott, A. M., Gullick, W. J., Ritter, G., Cohen, L., Scanlan, M. J., Cavanee, W. K., and Old, L. J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 639–644
23. Luwor, R. B., Johns, T. G., Murone, C., Huang, H. J., Cavenee, W. K., Ritter, G., Old, L. J., Burgess, A. W., and Scott, A. M. (2001) *Cancer Res.* **61,** 5355–5361
24. Mishima, K., Johns, T. G., Luwor, R. B., Scott, A. M., Stockert, E., Jungbluth, A. A., Ji, X. D., Suvarna, P., Voland, J. R., Old, L. J., Huang, H. J., and Cavenee, W. K. (2001) *Cancer Res.* **61,** 5349–5354
25. Hills, D., Rowlinson-Busza, G., and Gullick, W. J. (1995) *Int. J. Cancer* **63,** 537–543
26. Humphrey, P. A., Wong, A. J., Vogelstein, B., Zalutsky, M. R., Fuller, G. N., Archer, G. E., Friedman, H. S., Kwatra, M. M., Bigner, S. H., and Bigner, D. D. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4207–4211
27. Wikstrand, C. J., Hale, L. P., Batra, S. K., Hill, M. L., Humphrey, P. A., Kurpad, S. N., McLendon, R. E., Moscatello, D., Pegram, C. N., and Reist, C. J. (1995) *Cancer Res.* **55,** 3140–3148
28. Johns, T. G., Stockert, E., Ritter, G., Jungbluth, A. A., Huang, H. J., Cavenee, W. K., Smyth, F. E., Hall, C. M., Watson, N., Nice, E. C., Gullick, W. J., Old, L. J., Burgess, A. W., and Scott, A. M. (2002) *Int. J. Cancer* **98,** 398–408
29. Garrett, T. P., McKern, N. M., Lou, M., Elleman, T. C., Adams, T. E., Lovrecz, G. O., Zhu, H. J., Walker, F., Frenkel, M. J., Hoyne, P. A., Jorissen, R. N., Nice, E. C., Burgess, A. W., and Ward, C. W. (2002) *Cell* **110,** 763–773
30. Ferguson, K. M., Berger, M. B., Mendrola, J. M., Cho, H. S., Leahy, D. J., and Lemmon, M. A. (2003) *Mol. Cell* **11,** 507–517
31. Ogiso, H., Ishitani, R., Nureki, O., Fukai, S., Yamanaka, M., Kim, J. H., Saito, K., Sakamoto, A., Inoue, M., Shirouzu, M., and Yokoyama, S. (2002) *Cell* **110,** 775–787
32. Johns, T. G., Luwor, R. B., Murone, C., Walker, F., Weinstock, J., Vitali, A. A., Perera, R. M., Jungbluth, A. A., Stockert, E., Old, L. J., Nice, E. C., Burgess, A. W., and Scott, A. M. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 15871–15876
33. Elleman, T. C., Domagala, T., McKern, N. M., Nerrie, M., Lonnqvist, B., Adams, T. E., Lewis, J., Lovrecz, G. O., Hoyne, P. A., Richards, K. M., Howlett, G. J., Rothacker, J., Jorissen, R. N., Lou, M., Garrett, T. P., Burgess, A. W., Nice, E. C., and Ward, C. W. (2001) *Biochemistry* **40,** 8930–8939
34. Leahy, D. J., Dann, C. E., 3rd, Longo, P., Perman, B., and Ramyar, K. X. (2000) *Protein Expression Purif.* **20,** 500–506
35. Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989) *Gene* **77,** 61–68

36. Boder, E. T., and Wittrup, K. D. (1997) *Nat. Biotechnol.* **15,** 553–557
37. Meilhoc, E., Masson, J. M., and Teissie, J. (1990) *Bio/Technology* **8,** 223–227; Correction (1990) **8,** 768
38. Nice, E. C., and Catimel, B. (1999) *Bioessays* **21,** 339–352
39. Burgess, A. W., Cho, H. S., Eigenbrot, C., Ferguson, K. M., Garrett, T. P., Leahy, D. J., Lemmon, M. A., Sliwkowski, M. X., Ward, C. W., and Yokoyama, S. (2003) *Mol. Cell* **12,** 541–552
40. Arteaga, C. L., Ramsey, T. T., Shawver, L. K., and Guyer, C. A. (1997) *J. Biol. Chem.* **272,** 23247–23254
41. Decker, S. J. (1984) *Mol. Cell. Biol.* **4,** 571–575
42. Zhen, Y., Caprioli, R. M., and Staros, J. V. (2003) *Biochemistry* **42,** 5478–5492
43. Cho, H. S., and Leahy, D. J. (2002) *Science* **297,** 1330–1333
44. Cochran, J. R., Kim, Y. S., Olsen, M. J., Bhandari, R., and Wittrup, K. D. (2004) *J. Immunol. Methods* **287,** 147–158