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Research Paper

Domain-level antibody epitope mapping through yeast surface display of epidermal growth factor receptor fragments

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Abstract

Individual domains from extracellular proteins are potential reagents for biochemical characterization of ligand/receptor interactions and antibody binding sites. Here, we describe an approach for the identification and characterization of stable protein domains with cell surface display in *Saccharomyces cerevesiae*, using the epidermal growth factor receptor (EGFR) as a model system. Fragments of the EGFR were successfully expressed on the yeast cell surface. The yeast-displayed EGFR fragments were properly folded, as assayed with conformationally specific EGFR antibodies. Heat denaturation of yeast-displayed EGFR proteins distinguished between linear and conformational antibody epitopes. In addition, EGFR-specific antibodies were categorized based on their ability to compete ligand binding, which has been shown to have therapeutic implications. Overlapping EGFR antibody epitopes were determined based on a fluorescent competitive binding assay. Yeast surface display is a useful method for identifying stable folded protein domains from multidomain extracellular receptors, as well as characterizing antibody binding epitopes, without the need for soluble protein expression and purification.

Keywords: Yeast display; Monoclonal antibody; Epitope mapping; EGFR; Protein domain

1. Introduction

Yeast surface display has been developed as a method to affinity mature single-chain antibody fragments (Boder and Wittrup, 1997, 2000; Boder et al., 2000), and for engineering protein stability and expression (Shusta et al., 1999, 2000) through directed molecular evolution. It can also be used as a platform for screening a nonimmune human antibody library against a variety of hapten, peptide, and protein ligands (Feldhaus et al., 2003). We here extend yeast surface display to facilitate identification of stable, functional protein domains, and map antibody binding sites to individual domains, using the epidermal growth factor receptor (EGFR) as a model system.

Abbreviations: EGFR, epidermal growth factor receptor; mAb, monoclonal antibody; hEGF, human epidermal growth factor.

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The signaling pathways regulated by the epidermal growth factor (EGF) family of ligands and their receptors are responsible for directing the proliferation and differentiation of many different cell types (Carpenter and Cohen, 1990; Ullrich and Schlessinger, 1990). The EGFR is a membrane bound glycoprotein with a molecular weight of approximately 170 kDa. EGFR consists of a glycosylated external ligand-binding domain (621 residues), and a cytoplasmic domain (542 residues) connected by a short ~23 amino acid transmembrane linker (Ullrich et al., 1984). The EGFR contains 25 disulfide bonds, 12 N-linked glycosylation sites, and is generally considered to consist of four subdomains. Recent X-ray crystal structures of the EGFR have shown that the receptor subdomains interact to form a ligand-binding site (Garrett et al., 2002; Ogiso et al., 2002; Ferguson et al., 2003). In particular, domain I and domain III have been suggested to provide additive contributions for formation of a high-affinity ligand-binding site (Lax et al., 1991). Domains II and IV are cysteine rich, laminin-like regions that stabilize protein folding and contain a possible EGFR dimerization interface (Ferguson et al., 2003).

EGFR has been shown to be overexpressed on a wide variety of human malignancies, including squamous cell carcinoma, non-small-cell lung cancer, glioblastoma multiforme, and bladder, cervical, ovarian, kidney, and pancreatic cancers (reviewed in Hong and Ullrich, 2000; Mendelsohn and Baselga, 2000), and correlates with poor clinical prognosis (Nicholson et al., 2001), making it an important target for therapeutic intervention. Many EGFR-specific antibodies have been isolated, and some have demonstrated the ability to modulate tumor cell growth (Sato et al., 1983; Dean et al., 1994; Yang et al., 1999), while others have shown no effect on biological responses (Waterfield et al., 1982; Richert et al., 1983). In addition, there appears to be a correlation with the ability of an antibody to block ligand binding to the EGFR and inhibition of tumor growth or cell signaling (Gill et al., 1984; Dean et al., 1994; Yang et al., 1999). Methods to rapidly classify the biochemical properties of EGFR-specific antibodies would be a valuable research tool.

Previous approaches to antibody epitope mapping have involved expression of peptide fragments on the surface of bacteriophage (van Zonneveld et al., 1995; Yip et al., 2001, 2003; Itoh et al., 2003), or *Escherichia coli* (Christmann et al., 2001), and chemical synthesis of overlapping or mutated peptides (Wu et al., 1989; Yip et al., 2001, 2003), with subsequent antibody binding analysis. These methods were useful in mapping regions of antibody–antigen interaction, but in general can only be applied to antibodies that recognize a continuous linear epitope. Soluble, chimeric proteins have also been successfully used to map regions of antibody interaction (Cunningham et al., 1989), though this approach requires the availability of protein homologs that might not be obtainable for all systems. In addition, soluble protein fragments can be expressed or created by chemical or enzymatic proteolysis (Lax et al., 1988; Wu et al., 1989) and characterized for antibody binding. However, these methods are very laborious, and the soluble protein fragments must be individually characterized for proper folding, function and stability.

Using yeast surface display of EGFR fragments, we have rapidly characterized EGFR-specific antibodies based on receptor domain binding, recognition of linear or discontinuous epitopes, the ability to compete with ligand for receptor binding, and the presence of overlapping binding epitopes. In general, yeast express folded, functional proteins due to the protein folding and quality control machinery in the endoplasmic reticulum, by comparison to bacterial expression systems (Ellgaard and Helenius, 2003). EGFR fragments defined by stable protein breakpoints were expressed on the yeast surface and were properly folded, as assayed with conformationally specific EGFR antibodies. Tethering of proteins to the yeast cell surface obviates tedious, large-scale expression and purification of individual protein domains. Using a variety of commercially available and therapeutically relevant EGFR-specific antibodies, we demonstrate that yeast surface display can be used to rapidly screen for folded protein domains, as well as for identifying and characterizing antibody binding epitopes.

2. Materials and methods

2.1. 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 (Boder and Wittrup, 1997) by electroporation (Meilhoc et al., 1990) using a Bio-Rad (Richmond, CA) Gene Pulser Transfection Apparatus. The display plasmid is derived from pRS314 (Sikorski and Hieter, 1989) and contains the TRP1 marker that allows selection of the transformed yeast. Expression of EGFR proteins on the yeast cell surface was performed as previously described (Boder and Wittrup, 2000). Briefly, 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 OD_{600} of 5–6 was reached. Yeast cells were then induced for protein display by transferring to minimal media containing galactose, and incubated with shaking at 30 °C for 24 h. Cultures were then stored at 4 °C until analysis.

2.2. Antibody labeling experiments on the yeast cell surface

Raw ascites fluid containing the c-myc monoclonal antibody (mAb) 9E10 was obtained from Covance (Richmond, CA). The anti-human EGFR mouse monoclonal antibodies H11 (Wikstrand et al., 1995), 111.6, B1D8 (Kudlow et al., 1984), 225 (Sato et al., 1983), 528 (Sato et al., 1983), and 199.12 were purchased from LabVision (Fremont, CA), and EGFR1 (Waterfield et al., 1982) from Biodesign International (Saco, ME). The anti-human EGFR rat monoclonal antibody ICR10 (Modjtahedi and Dean, 1993) was purchased from Abcam (Cambridge, UK). The anti-human EGFR mouse monoclonal antibodies 10B7, 15E11, and 2D2 (Baron et al., 1997) were graciously provided by the Nita J. Maihle of the Mayo Cancer Center.

A total of 1×10^6 yeast cells were washed with FACS buffer (phosphate-buffered saline containing 1 mg/ml bovine serum albumin) and incubated with either anti-cmyc ascites (1:50 dilution), or the appropriate 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, or FITC-labeled anti-rat 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 was obtained with a Coulter Epics XL flow cytometer (Beckman-Coulter), and further 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 prior to labeling with antibodies.

2.3. Human epidermal growth factor (hEGF) competition binding experiments

hEGF from PeproTech was used without further purification. The affinities of mAbs 225, 111.6, H11, and 15E11 for the yeast displayed EGFR fragment 273–621 were determined by flow cytometry as described above, using varying concentrations of mAbs. The specificity of hEGF binding to EGFR 273–621 was determined by competition binding with the mAbs 225, 111.6, H11, 15E11, and 528. The concentrations of mAbs were fixed around $K_{D,app}$ values, i.e., 225 = 0.4 μg/ml; 111.6, H11, and 15E11 = 0.6 μg/ml, and equilibrium binding was measured at various concentrations of hEGF. The experimental conditions were identical to those described above, except that mAbs and hEGF were incubated with yeast at room temperature for 1.5 h with shaking.

2.4. Data analysis

The binding interaction between mAbs and the surface displayed EGFR fragment 273–621 was assumed to be a single site binding model with no ligand depletion. The mean fluorescence intensity was normalized by maximal and minimal mean fluorescence intensities to obtain the fraction of mAbs binding to the surface displayed EGFR 273–621. The apparent dissociation constant ($K_{D,mAb}$) was obtained by fitting the data using the following equation on SigmaPlot 2001 software (SPSS Inc.) (Stein et al., 2001).

$$f_{mAb} = \frac{[mAb]}{[mAb] + K_{D,mAb}} \quad (1)$$

where f_{mAb} is the fraction of mAb bound to the EGFR fragment 273–621, [mAb] is the concentration of antibody, and $K_{D,mAb}$ is the apparent dissociation constant of the mAb. For the EGF competition bind-

ing assays, it was assumed that hEGF and the mAbs compete for a single binding site. The following equation was used to fit the competition binding data (Limbird, 1996; Stein et al., 2001).

$$f_{\mathrm{mAb}} = \frac{1}{1 + 10^{(\log[\mathrm{hEGF}] - \log[\mathrm{EC}_{50}])}} \quad (2)$$

where f_{mAb} is the fraction of mAb bound to the EGFR 273–621 in the presence of hEGF, [hEGF] is the concentration of hEGF, and [EC_{50}] is the concentration of hEGF that reduces binding of the mAb by 50% of its maximum. Using [EC_{50}] and $K_{\mathrm{D,mAb}}$, the affinity of hEGF ($K_{\mathrm{D,EGF}}$) for the EGFR fragment 273–621 was estimated using the following equation (Cheng and Prusoff, 1973; Stein et al., 2001).

$$K_{\mathrm{D,hEGF}} = \frac{\mathrm{EC}_{50}}{1 + \frac{[\mathrm{mAb}]}{K_{\mathrm{D,mAb}}}} \quad (3)$$

where [mAb] is the fixed mAb concentration used in the competitive binding assay.

2.5. Determination of overlapping antibody epitopes

Biotinylated 111.6 and 528 antibodies were purchased from LabVision. To fluorescently label the mAbs H11 and 111.6, an Alexa Fluor 488 monoclonal antibody labeling kit (A-20181) was purchased from Molecular Probes (Eugene, OR), and used according to the manufacturer's protocols. Yeast cells expressing EGFR 294–543 were incubated with 10 μg/ml biotinylated (528, 111.6) or Alexa-488 labeled (H11, 15E11) mAb and 50 μg/ml unlabeled competitor mAb for 30 min at 4 °C. Cells were washed with ice-cold FACS buffer and incubated for 20 min at 4 °C with streptavidin–phycoerythrin (for biotinylated samples) or directly analyzed by flow cytometry.

3. Results

3.1. Design and yeast surface display of EGFR fragments

The extracellular portion of the EGFR consists of four receptor subdomains that interact to form a ligand binding site (Garrett et al., 2002; Ogiso et al., 2002; Ferguson et al., 2003). Previously, in the absence of high-resolution structures of the EGFR or EGF/EGFR co-complex, biochemical characterization has been used to investigate the nature of the ligand/receptor interactions (Lax et al., 1988, 1990, 1991; Kohda et al., 1993). We have generated fragments of the EGFR to probe minimal folded receptor protein domains using yeast surface display. EGFR fragments were designed in part from published data on a receptor deletion in the case of EGFR 1–124 (Lax et al., 1990), proteolytic cleavage for EGFR 302–503 (Kohda et al., 1993), chemical cleavage for EGFR 1–294 and EGFR 294–543 (Lax et al., 1988), and a naturally occurring receptor truncation expressed on tumor cells for EGFR 273–621 (Sugawa et al., 1990) (Fig. 1A). As our work was initiated prior to published structural information, comparative modeling

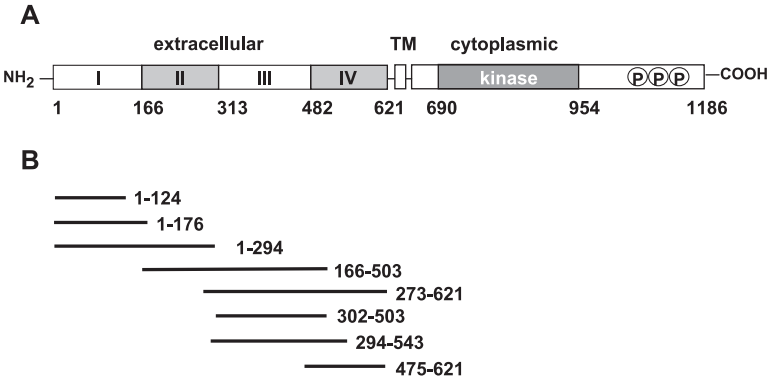

Fig. 1. Design of EGFR fragments for domain mapping. (A) Full-length EGFR protein indicating extracellular, transmembrane (TM), and cytoplasmic kinase domains. (B) EGFR truncations attempted to be displayed on the surface of yeast.

of the EGFR based on the insulin-like growth factor 1 (Jorissen et al., 2000) also helped to define some of the fragment breakpoints. This modeling suggested that an intact domain I and/or domain III with the presence of a portion of the next domain (domain II: Trp 176 or domain IV: Trp 492) was required for proper protein folding.

EGFR fragments encompassing domains I, II, III, and IV (Fig. 1B) were subcloned into the yeast display system for cell surface expression. Plasmid DNA encoding for the amino acid sequence of the EGFR fragments described in Fig. 1 was transformed into the yeast strain EBY100 (Boder and Wittrup, 1997). The yeast display constructs included N-terminal hemagglutinin and C-terminal c-myc epitope tags flanking the EGFR inserts for detection and quantitation of cell surface proteins. Yeast cultures were grown at 30 °C to an OD_{600} of 5, and then induced for expression by the addition of galactose to the culture medium. Typically, optimal EGFR protein expression occurred after induction for 24 h at 30 °C. Induced yeast cultures were assayed for cell surface protein expression through indirect immunofluorescence of the C-terminal c-myc epitope tag as measured by flow cytometry. EGFR fragments 1–124, 1–176, 1–294, 273–621, and 294–543 were all well expressed on the yeast cell surface, as indicated by reactivity of the c-myc tag with the mAb 9E10 (Fig. 2). The EGFR fragment 475–621 was not as well expressed as the other fragments, as demonstrated by the lower mean fluorescence histogram for c-myc labeling (Fig. 2). The EGFR fragments 166–503 and 302–503 were not expressed on the cell surface, suggesting that these fragments corresponded to unstable protein domain breakpoints that could not be properly folded or trafficked though the yeast secretory pathway (Ellgaard and Helenius, 2003). Full-length EGFR extracellular domain (EGFR 1–621) and receptor fragments missing most of domain IV (EGFR 1–501, EGFR 1–520) were also expressed on the yeast cell surface (data not shown), but were not useful for domain epitope mapping and were omitted from all studies described below.

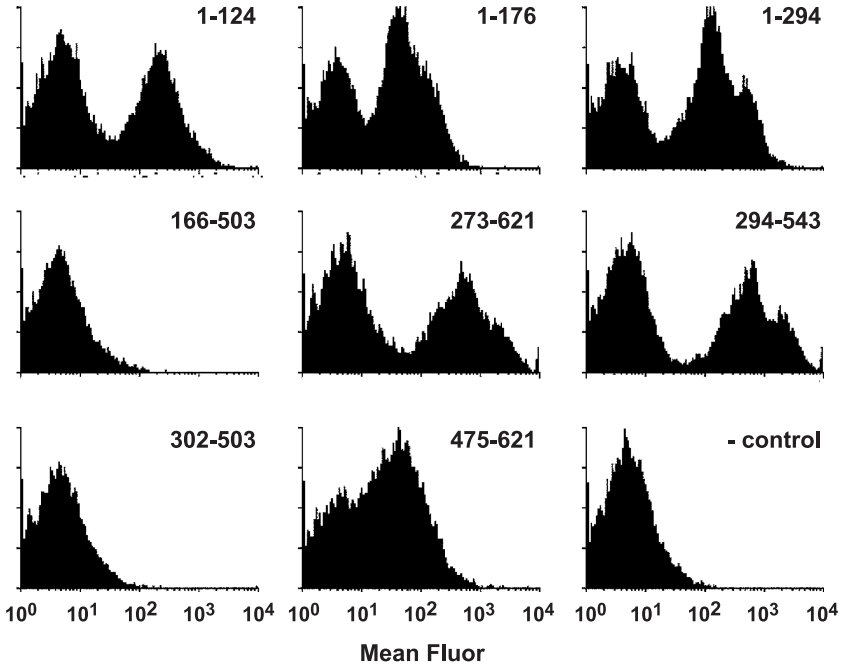

Fig. 2. Display of EGFR fragments on the yeast cell surface. Flow cytometry histograms depicting the mean fluorescence signal of antibody labeling of the C-terminal c-myc epitope tag of yeast-displayed EGFR fragments. The presence of the c-myc tag confirms the expression of the full-length polypeptide on the yeast cell surface. With the yeast display system, a percentage of cells do not express protein on their surface, resulting two histogram peaks, which also provides a good internal negative control for labeling.

3.2. Antibody domain mapping using yeast displayed EGFR fragments

EGFR-specific antibodies were used to assay protein expression and to map binding domains of the EGFR fragments displayed on yeast. In some cases, the general region of antibody reactivity was known previously. mAbs 225 and 528 were known to bind to domain III of the EGFR, while mAbs 10B7, 15E11, and 2D2 were raised against the linear epitopes 290–311, 352–369, and 556–567 of the EGFR respectively (Baron et al., 1997). In other cases, as for mAbs H11, 111.6, B1D8, ICR10, 199.12, and EGFR1, the exact EGFR binding domain had not been reported previously.

A matrix of antibodies was tested for reactivity with all of the displayed EGFR fragments, using flow cytometry to measure indirect immunofluorescence of yeast cells (Fig. 3 and Table 1). Using this information, we were able to localize antibody binding domains on the EGFR protein. As shown in Table 1, mAbs ICR10 and 199.12 bind to domain I of the EGFR, between residues 124–176. The mAb EGFR1 was shown to bind to domain II of the receptor, between residues 176–294. As expected, mAbs 225 and 528 bind to domain III of the receptor, between 294 and 475. mAbs H11, 111.6, 15E11, and B1D8 also bind to domain III of the receptor, between residues 294–475. mAb 2D2, raised against the linear peptide epitope 556–567 (Baron et al., 1997), bound strongly to EGFR 273–621, but not 294–543, demonstrating reactivity for domain IV (Table 1). Binding of 2D2 to the EGFR fragment 475–621 was weak, but the lower fluorescence signal could be due to the fact that EGFR 475–621 was not as well expressed on the yeast surface as the other EGFR receptor fragments (Fig. 2).

It should be noted that the antibody binding epitope may flank the breakpoint of the particular EGFR protein fragment tested, generating a false negative result. mAb 10B7, which was raised against the linear peptide epitope 290–311 of the EGFR (Baron et al., 1997), bound to the EGFR fragment 273–621 and exhibited slight binding to EGFR 1–294, but did not bind to EGFR 294–543. These results suggest that the

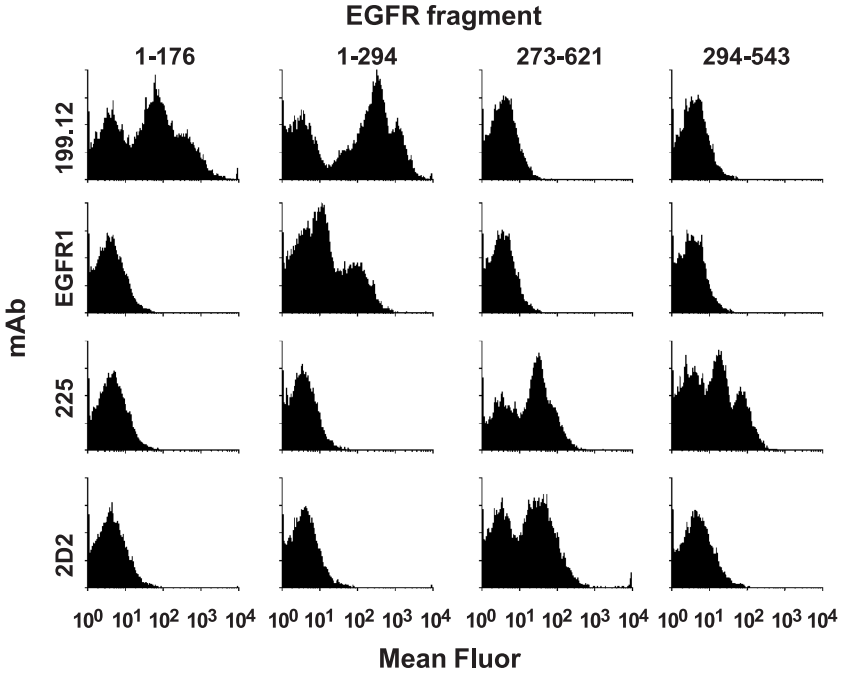

Fig. 3. Antibody domain mapping of yeast-displayed EGFR fragments. Representative flow cytometry histograms depicting the mean fluorescence signal of EGFR antibody labeling of yeast displayed EGFR fragments. EGFR antibodies do not bind to the uninduced yeast negative control. Data is representative of several experiments.

Table 1
Summary of EGFR mAb domain mapping experiments

	1–124	1–176	1–294	294–543	273–621	475–621	Epitope	EGF comp
ICR10	–	++	++	–	–	–	Conf	NT
199.12	–	++	++	–	–	–	Conf	NT
EGFR1	–	–	++	–	–	–	Conf	NT
H11	–	–	–	++	++	–	Linear	No
B1D8	–	–	–	+	+	–	Conf	NT
111.6	–	–	–	++	++	–	Linear	Yes
225	–	–	–	++	++	–	Conf	Yes
528	–	–	–	++	++	–	Conf	Yes
15E11	–	–	–	++	++	–	Linear	No
10B7	–	–	+	–	+	–	Linear	NT
2D2	–	–	–	–	++	+	Linear	NT

(–) = No binding. (+) = Weak, but detectable binding. (++) = Strong binding. NT = Not tested.

major binding contribution in this epitope encompasses residues 290–294, however residues beyond 294 might also be involved in 10B7 antibody binding.

3.3. Linear versus conformationally specific EGFR antibody epitopes

The antibodies in this study were further categorized for conformational or linear epitopes using heat denaturation of the EGFR proteins tethered to the yeast cell surface. Yeast cells displaying EGFR fragments were heated at 80 °C for 30 min, chilled on ice, and then assayed for binding against the panel of mAbs from Table 1. The detection of the C-terminal c-myc tag with the mAb 9E10 confirmed the presence of full-length EGFR fragments on the yeast cell surface, indicating that the proteins and cells were not compromised during the heat treatment (Fig. 4). It

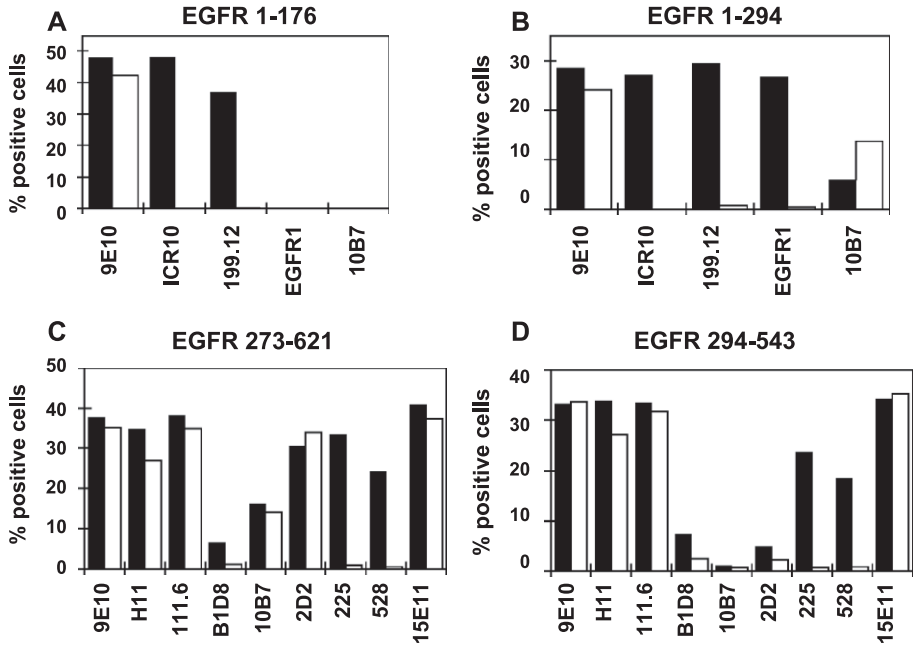

Fig. 4. Linear versus conformational EGFR-specific mAb epitopes. mAb labeling was assayed before (solid bars) and after (open bars) protein denaturation by heating yeast cells at 80 °C for 30 min. 9E10 mAb labeling (specific for the linear C-terminal c-myc tag) demonstrates that heat treatment does not compromise yeast surface displayed EGFR proteins.

is possible that the yeast-displayed proteins could refold on the cell surface following denaturation. Antibodies that were known to bind to EGFR with conformational specificity (mAbs 225, 528, and EGFR1) were used to confirm the denaturation of yeast-displayed proteins upon heat treatment. Heat denaturation abolished the EGFR binding of mAbs ICR10, 199.12, EGFR1, 225, 528, and B1D8, indicating that these antibodies are reactive towards conformational or discontinuous epitopes of the receptor. mAbs H11, 111.6, 10B7, 15E11, and 2D2 all retained their binding reactivity upon heat treatment of yeast displayed EGFR fragments, and in some cases antibody binding increased upon protein denaturation (Fig. 4 and data not shown), indicating that these antibodies are specific for linear or continuous epitopes. This data is in agreement with the fact that 10B7, 15E11, and 2D2 were all raised from immunization of mice with linear peptides (Baron et al., 1997).

3.4. Competition binding of hEGF to yeast displayed EGFR 273–621

Previously, EGFR-specific antibodies have been categorized based on their ability to compete ligand binding. It has been shown that competition of ligand binding to the EGFR on tumor cells has important therapeutic implications for blocking cell signaling (Gill et al., 1984; Dean et al., 1994; Yang et al., 1999). The mAbs used in this study can be assayed for ligand-binding competition using EGFR fragments displayed on the yeast cell surface. The EGFR fragment 273–621 was used for this purpose, as it has been shown that the majority of hEGF binding results from interaction with domain III of the EGFR (Lax et al., 1988; Kohda et al., 1993). Equilibrium binding curves for mAb binding to yeast-displayed EGFR 273–621 were obtained by indirect immunofluorescence using flow cytometry (Fig. 5A). The apparent dissociation constants (K_D) of mAbs for EGFR 273–621 were 0.96 ± 0.11 nM (225), 3.94 ± 1.22 nM (111.6), 4.00 ± 0.14 nM (H11), and 17.15 ± 2.19 nM (15E11). This is in agreement with published values for mAb 225 binding to A431 carcinoma cells (~ 1 nM), HeLa cells (1.1 nM), and human fibroblasts (1 nM) (Sato et al., 1983).

Binding assays were performed through competition of the mAbs 225, 528, 111.6, 15E11, and H11

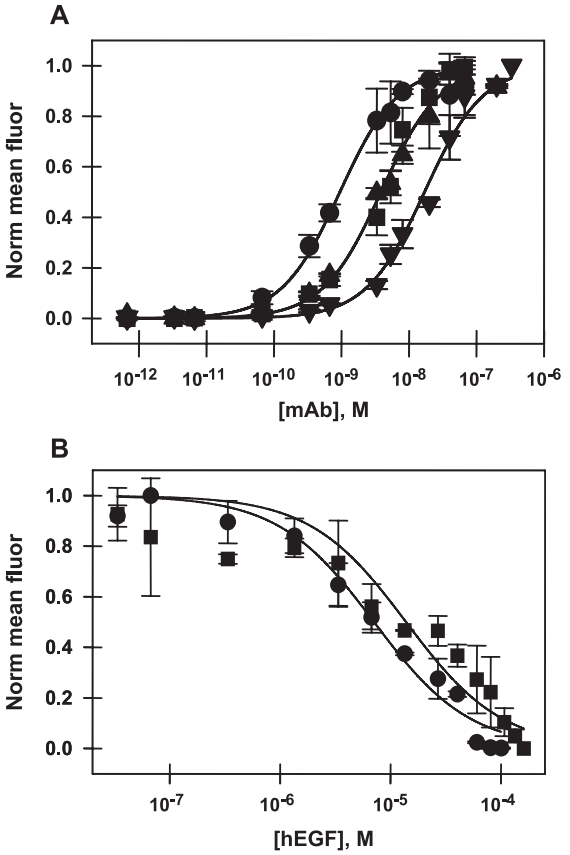

Fig. 5. hEGF competition binding of mAbs to yeast-displayed EGFR 273–621. (A) The affinities of mAbs 225 (●), 111.6 (■), H11 (▲), and 15E11 (▼) for the yeast-displayed fragment EGFR 273–621 were determined by flow cytometry using indirect immunofluorescence. (B) The ability of hEGF ligand to block antibody interaction with EGFR 273–621 was determined by competition binding using flow cytometry. Error bars indicate the standard error from duplicate experiments.

with increasing concentrations of hEGF. At high concentrations, hEGF inhibited mAbs 225, 528, and 111.6 binding (Fig. 5B and data not shown). Based on this competition data, equilibrium binding constants can be calculated for the interaction between hEGF and EGFR 273–621 displayed on yeast ($K_{D,EGF} = 1.92 \pm 0.11$ μM and 7.00 ± 0.85 μM for 225 and 111.6, respectively). These values are within several-fold of what has been previously shown for hEGF binding to the soluble extracellular domain of the EGFR (Lemmon et al., 1997; Elleman et al., 2001). The slight decrease in affinity we observed may be

due to the absence of domains I and II from the EGFR protein (Lax et al., 1990), or possible hyperglycosylation of the yeast EGFR, as previously seen with the binding of erythropoeitin to its receptor (Zhan et al., 1999). hEGF did not compete for the binding of mAbs H11 and 15E11 even at the highest concentration tested (data not shown). Overall, this binding data confirms published results for antibody inhibition of hEGF binding to receptor (Sato et al., 1983; Gill et al., 1984).

3.5. Determination of overlapping antibody epitopes

In addition to classifying EGFR-specific antibodies based on their reactivity to linear or conformational epitopes and their ability to block ligand binding, the presence of overlapping epitopes was assayed using cross-blocking experiments. mAbs H11, 15E11 were fluorescently labeled with Alexa-488 (Molecular Probes) through free amino groups, while mAbs 528 and 111.6 were purchased as biotinylated conjugates (LabVision). Flow cytometry was used to measure the competition binding of labeled antibody to the yeast-displayed EGFR fragment 293–543 with a five-fold excess of unlabeled antibody. mAbs 225 and 528 were shown to cross-block one another in binding to EGFR 294–543 (Fig. 6A). This result confirms previously reported data demonstrating overlapping binding epitopes with 225 and 528 mAbs (Sato et al., 1983). In addition, the mAbs H11, 15E11, and 111.6 were shown to cross-block one another, but not mAbs 528 or 225 (Fig. 6A) indicating that these antibodies have their own overlapping epitope.

A

Competitor antibody \ Fluorescent antibody	528	H11	111.6	15E11
528		+	+	+
225	-	+	+	+
H11	+		-	-
111.6	+	-		-
15E11	+	-	-	

B

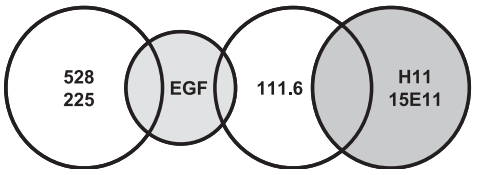

Fig. 6. Determination of overlapping EGFR-specific mAb epitopes. (A) Antibody cross-blocking was determined by competitive binding of biotinylated (528, 111.6) or Alexa-488 labeled (H11, 15E11) mAb and unlabeled competitor mAb to yeast cells displaying EGFR 294–543. Cells were washed and incubated with streptavidin–phycoerythrin (for biotinylated samples) or directly analyzed by flow cytometry (for fluorescent samples). A fluorescent signal, (+), indicates that the antibodies did not compete one another for binding to EGFR. Lack of a fluorescent signal, (–), indicates inhibition of binding to EGFR by unlabeled competitor antibody. (B) Diagram of overlapping EGFR domain III specific mAb epitopes.

4. Discussion

This work describes a novel method of expressing and analyzing single protein domains using yeast surface display technology. It is generally found that only properly folded proteins will pass through the stringent quality control system of the eucaryotic secretory apparatus (Ellgaard and Helenius, 2003). Yeast display was used to rapidly probe the expression of stable, folded domains of the EGFR. Tethering of EGFR fragments to the yeast cell surface removed the need for soluble expression, purification, and characterization of individual proteins domains, which is laborious and time consuming, and not guaranteed to produce useable protein fragments for analysis. Fragments ending in a breakpoint at residue 503 were not successfully expressed, implying that truncation at this site results in aberrant protein folding. The EGFR fragment 1–501 is well expressed on the yeast surface (data not shown), and in mammalian systems (Elleman et al., 2001), suggesting that a terminal free cysteine at residue 502 that results upon receptor cleavage has a deleterious effect on protein folding and expression.

Antibody domain mapping and binding epitope characterization can be performed using the yeast displayed EGFR protein fragments. Several commercially available and clinically relevant EGFR-specific antibodies were mapped for their region of receptor interaction, and characterized for linear versus con-

formationally specific binding epitopes. The antibodies were also classified for their ability to compete ligand binding, and for the presence of overlapping epitopes. It is important to note that once the yeast display system of EGFR fragments was established, the domain mapping and characterization were rapid and could be carried out over the course of a few days, in comparison with biochemical and biophysical analyses using soluble protein domains.

None of the 11 antibodies analyzed bound to the EGFR fragment 1–124. This particular region of the EGFR may not be immunogenic, as evidenced from the failure of one study to raise antibodies against the linear peptide epitope 77–93 (Baron et al., 1997). However, in the absence of conformationally specific antibodies against this receptor domain, we cannot rule out the possibility that this fragment may not be functional when expressed on the yeast cell surface. mAbs 225 and 528 are both conformational antibodies, while mAbs H11 and 15E11, and 111.6 are reactive against linear regions, and these antibodies share respective epitopes with one another as evidenced by the cross-blocking experiments. Even though only a small subset of EGFR-specific antibodies were tested in this study, it is intriguing to speculate that there are at least two major regions of receptor immunodominance within EGFR domain III that arises from immunization with antigens.

Since the mAb 15E11 was raised against a linear peptide corresponding to residues 352–369 of the EGFR, it is suggestive that mAbs H11 and 111.6 bind to a similar region of the receptor (Fig. 6), however, only mAb 111.6 competes with hEGF. The proteins might possess a similar binding epitope, but other mechanisms could be responsible for competition binding of antibodies and/or hEGF on the yeast cell surface. Considering the size of an intact IgG molecule (MW ~ 150 kDa) compared to hEGF (MW ~ 6 kDa), steric hindrance could occur, which would occlude binding due to spatial constraints. In addition, conformational changes induced through protein binding could abolish the binding site of another molecule.

The EGFR and its family members were found to be overexpressed on a variety of human malignancies (Hong and Ullrich, 2000; Mendelsohn and Baselga, 2000), making them a relevant therapeutic target for antibody intervention. Many EGFR-specific antibodies, including mAbs 225 and 528 (Sato et al., 1983; Masui et al., 1984), have been shown to inhibit the growth of cancer cells in vitro and in vivo, while other mAbs, like EGFR1, have no effect on cell proliferation (Waterfield et al., 1982). There appears to be a correlation with the ability of an antibody to block ligand binding to receptor and inhibition of tumor growth (Gill et al., 1984; Dean et al., 1994; Yang et al., 1999). However, this is not the only determinant of therapeutic efficacy, as Herceptin, an antibody currently in use for breast cancer treatment, binds to the juxtamembrane region of HER2 (an EGFR family member) (Cho et al., 2003). Therefore, methodology for characterizing EGFR-specific antibody binding epitopes is a useful tool for classifying panels of clinically relevant antibodies.

Previous methods of antibody epitope mapping using biosynthetic systems involved the presentation of protein or peptide fragments on the surface of bacteriophage (van Zonneveld et al., 1995; Yip et al., 2001, 2003; Itoh et al., 2003), or *E. coli* (Christmann et al., 2001), and measuring antibody binding. In addition, overlapping (Wu et al., 1989; Yip et al., 2001) or mutated (Yip et al., 2003) synthetic peptides have been used to map antibody binding epitopes. While these methods were successful in defining linear or continuous regions of antibody interactions, they are less useful for characterizing antibodies that are reactive towards a discontinuous binding epitope. More recently, conformational epitopes were successfully detected using phage display (Yip et al., 2003), and the yeast-displayed presentation of a cancer/testis antigen (Mischo et al., 2003) was useful for serological detection of antibodies in cancer patients. Homolog scanning mutagenesis has also been used to create a set of chimeric human growth hormones to successfully map antibody and receptor interactions (Cunningham et al., 1989). While this method was an elegant demonstration of the use of mutagenesis to characterize mAb binding and blocking of the hormones to its receptor, it requires the availability of protein homologs and secretion of functional chimeras in the *E. coli* periplasm. By contrast, our system involves a simple protocol of cell surface expression and immunofluorescent labeling. The analysis requires the use of a flow cytometer or other method of measuring cell fluorescence, but can be performed immediately without the need to purify and characterize individual protein fragments.

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