Identification of peptides mimicking the natural epitope recognized by the CB-EGF1 anti-EGF monoclonal antibody

RESEARCH

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ABSTRACT

As part of an integral structure and function study, we characterized the natural epitope on the EGF recognized by the CB-EGF1 monoclonal antibody, by using peptides displayed on the coat protein of filamentous bacteriophages. Data analyzed demonstrated that the CB-EGF1 monoclonal antibody recognizes a conformational epitope, comprising aminoacids S9, H10, Y13, K28, E40 and R41. The identified peptide sequences are mimicking the region on the EGF interacting with the β-chain of the EGF receptor (EGFR). Synthetic peptides F22-54 and F22-55 compete with the EGF for binding to the CB-EGF1 antibody, as demonstrated by ELISA, but lacking biological activity in the EGF-EGFR system.

Keywords: EGF, EGFR, phage display, combinatorial libraries

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RESUMEN

Identificación de péptidos miméticos al epítopo reconocido por el anticuerpo monoclonal específico por el EGF, CB-EGF1. Como parte de un estudio integral de estructura y función, se caracterizó el anticuerpo monoclonal CB-EGF1, específico por el EGF, con el empleo de la tecnología de presentación de péptidos en la cápsida de los bacteriófagos filamentosos. Los datos de este trabajo demuestran que el anticuerpo CB-EGF1 reconoce un epítopo conformacional en la molécula de EGF compuesto por los aminoácidos S9, H10, Y13, K28, E40 y R41. Las secuencias de péptidos identificadas mimetizan la zona de interacción del EGF con la cadena beta del REGF. Los péptidos sintéticos F22-54 y F22-55 compiten con el EGF por la unión al anticuerpo CB-EGF1, según se demostró mediante ELISA, pero no presentan actividad sobre el sistema EGF-REGF.

Palabras clave: EGF, REGF, presentación en fagos, bibliotecas combinatorias

*I*ntroduction

Epitopes recognized by monoclonal antibodies (MAb) have been traditionally identified by screening peptide libraries of antigen fragments [1, 2], or libraries of synthetic peptides or complementary DNA fragments expressed *in vitro* [3]. An advantageous alternative to these immunochemical and biological techniques comprises libraries of peptides displayed on the surface of filamentous bacteriophages. Although low recovery yields are achieved by this procedure during selection (down to the single particle level, in theory), selected peptides are identified and characterized [4]. Those peptides mimicking the natural antigen are named phagotopes.

The epidermal growth factor (EGF) is a polypeptide regulating growth [5], with a stimulating effect on epidermal proliferation and also inhibiting gastric acid secretion [6]. This peptide of 53 aa. (6045 Da) shows a high-affinity binding to the extracellular domain of specific cell surface receptors, named EGF receptors (EGFRs) [7]. This interaction is essential for activating the EGFR tyrosine kinase domains, triggering signal transduction that ultimately results in DNA synthesis and cell proliferation [6, 8]. EGFR activation is relevant for developing proliferative, mature, survival, apoptotic, angiogenic and metastatic cellular phenotypes [6]. Due to the relevance of the EGF-EGFR system in the pathogenesis of several cancers, the search for agents selectively inhibiting their interaction has became one of the most important challenges, with antibodies and tyrosine kinase inhibitors included among them [9, 10].

The binding sites and the detailed specificity of the CB-EGF1, an anti-EGF neutralizing monoclonal antibody previously obtained in the Center for Genetic Engineering and Biotechnology of Havana (CIGB), were characterized [11] as a part of integral structure and function studies of several molecules.

To map the epitope recognized by this MAb in the EGF molecule, two libraries of linear (pVII-9aa) and cyclic (pVIII-9CaaC) peptides respectively were screened. Both libraries bear peptides displayed on the surface of f1 filamentous bacteriophages, fused to the pVIII major coat protein [12].

The natural epitope recognized by, and peptides with binding activity to, the CB-EGF1 MAb were identified. These peptides were evaluated in CB-EGF1 binding assays and EGF biological activity experiments *in vitro*.

*M*aterials and methods

Screening of phage displayed-peptide libraries

Peptides were selected from the pVIII-9aa and pVIII-9CaaC libraries by a biopanning procedure [13], modified from the former technique described by Felici *et al* [12]. Polystyrene beads (Ø 6.4 mm, Precision Plastic Ball Co., Chicago, USA) were coated with

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the CB-EGF1 MAb at 10 mg/mL in coating solution (50 mM sodium carbonate, pH 9.6), and incubated overnight at 4 °C. Beads were blocked for 4 h at 4 °C with bovine serum albumin (BSA) diluted to 10 mg/mL in phosphate buffered saline (PBS). After repeated washing with PBS containing 0.5% Tween 20 (PBS-Tween 20), beads were pre-incubated with aproximatedly 3 x 10^{10} M13KO7 kanamycin resistance-transducing units (Kan^R-TU) in 1 mL of PBS-Tween 20 containing 1 mg/mL BSA (PBS-Tween 20/BSA) for 4 h at 4 ºC. Approximately 3.2 x 10^{10} ampicillin resistance-transducing units (Amp^R-TU), either from the pVIII-9aa or the pVIII 9CaaC libraries, were added during the first round of selection. Mixes were agitated overnight at 4 ºC. Unbound fractions were discarded and beads were repeatedly washed with PBS-Tween 20. Adsorbed fractions were recovered by incubating with a solution containing 0.1 M HCl and 1 mg/mL BSA, pH 2.2. The eluted phage mixes were immediately neutralized with 2M Tris, pH 9.0 and further enriched by repeated rounds of selection.

Immunochemical identification of individual clones

For obtaining individual phage preparations, 0.4 mL cultures of *E. coli* TG1 cells were infected with 10^4 Amp^R-TU of recovered phage particles and immediately superinfected with 10^{11} Kan^R-TU of the M13KO7 helper phage, and grown in LB medium supplemented with 70 mg/mL Kan, 100 mg/ml Amp and 0.1 mM IPTG for 5 h at 37 ºC under vigorous shaking. Cultures were stopped, centrifuged and phage particles were precipitated from supernatants by adding 4% polyethylene glycol MW 8000 in 0.5 M NaCl. Positive clones were subsequently characterized by an anti-phage ELISA, by using the CB-EGF1 Mab for capturing particles and a peroxidase-conjugated anti-M13 antibody for their detection.

DNA sequencing

Nucleotide sequences of phage clones were determined according to the dideoxynucleotide method [14], with the -40 sequencing primer of the M13mp-series vectors.

Peptide synthesis

Peptides F22-54, F22-55 and F21-34 were synthetized in the Chemical Synthesis Department at the CIGB.

Immunochemical recognition of synthetic peptides F22-54 and F22-55 by the MAb CB-EGF1

All steps were carried out in a volume of 100 µL per well if not specified otherwise. Polystyrene plates (High Binding, Costar, USA) were incubated with 2% glutaraldehyde for 2 h at 37 ºC. Plates were coated with F22-54 and F22-55 peptides at 10 mg/mL each in coating solution, incubated overnight at 4 ºC in a humid atmosphere, and subsequently washed with PBS-Tween 20 and blocked with PBS-Tween 20 containing 2% BSA for 2 h at 37 ºC. Afterwards, plates were washed and the CB-EGF1 MAb was added at a 1:1000 dilution. Following a 1 h incubation at 37 ºC, plates were washed again and an anti-mouse peroxidase-conjugated antibody (Amersham Biosciences) was added at a 1:5000 dilution. After washing, positive reactions were visualized with 1 mg/ml o-phenylene diamine in 0.1 M of citric acid, 0.2 M Na_2HPO_4 pH 5.0, and 0.3% H_2O_2 as substrate; reactions were stopped with 50 µL of 2.5 M H_2SO_4. Optical densities at 492 nm (O.D.) were measured in a MicroELISA Multiskan System plate reader (Titerek, Helsinki, Finland). Duplicate samples were analyzed. The assay was validated by using human recombinant EGF (hrEGF) as the positive control, and an unrelated peptide (F21-34) and blank as baselines.

Competition ELISA

Assay conditions were previously established. All steps were carried out in a volume of 100 µL per well if not specified otherwise. Polystyrene plates (High Binding, Costar, USA) were coated with 125 ng/mL hrEGF in coating solution, and incubated overnight at 4 ºC in humid atmosphere. After washing with PBS-Tween 20, they were blocked with PBS-Tween 20 containing 2% BSA and incubated for 2 h at 37 ºC in a humid atmosphere. In the mean time, MAb-peptide mixes were prepared by incubating CB-EGF1 samples (1:20000) for 2 h at room temperature with 10 mg of each peptide (F22-54 or F22-55, respectively). Plates were washed again and incubated with MAb-peptide mixes for 1 h at 37 ºC. Positive reactions were visualized, stopped and measured as described above. The CB-EGF1 MAb that had not been incubated with peptides was used as a positive control.

Cell proliferation assay

Briefly, cell culture 96-well plates were seeded with 3T3 A31 murine fibroblast cells (ECACC) at 15 000 cells per well. After 24 h, the cell culture medium was discarded; cells were washed twice with PBS, followed by adding fresh serum-free culture medium and incubated for another 24 h. The culture medium was removed and different doses of the peptides and hrEGF reference (hrEGF-0601, CIGB, Havana, Cuba) for the assay were added and incubated for 24 h. Plates were stained with crystal violet for 3 min and washed with tap water after stain removal. The staining was dissolved by adding 100 µL of a 10% acetic acid solution. Absorbance was determined at 578 nm.

Statistical analysis

Results from peptide immunochemical recognition by the CB-EGF1 MAb, competition ELISA and cell proliferation assays were expressed as the mean ± standard deviation values. Duncan's Multiple Range Test was used for analyzing the statistical significance of competition ELISA data. Statistically significant values corresponding to $p<0.05$ were denoted (*).

Results and discussion

The CB-EGF1 MAb is a murine IgG1 subclass antibody, with neutralizing activity against the EGF molecule on its native conformation [11]. This MAb was used to capture filamentous phages from the phage-displayed peptide libraries pVIII-9aa and pVIII-9CaaC. Both libraries showed increased amounts of phages adsorbed from the first to the third rounds of selection (Table 1), denoting specific am-plification of phages recognized by the MAb.

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Table 1. Titers of fractions adsorbed during selection rounds (Amp^R-TU/mL).

Library	Selection rounds		
	1	2	3
pVIII9aa	1.4×10^6	1.3×10^7	4.9×10^8
pVIII9CaaC	8.0×10^5	1.2×10^6	2.0×10^7

The binding capacity of individual phage clones was confirmed by an anti-phage ELISA, and nucleotide sequences of positive clones were determined. These 27 bp-long DNA sequences were translated and peptides displayed in the amino terminus of the pVIII protein were identified (Table 2), with the SKFDR linear peptide established as the consensus after alignment.

Molecular modeling of the EGF structure confirmed the presence of these residues within a 10 Å region. Tyrosine (Y) to phenyl-alanine (F) and glutamic acid (E) to aspartic acid (D) substitutions were found in these clones. These are the most common changes observed in this type of studies since identified peptides not exactly match the natural antigen. This was also derived from the lateral chain and electric charge similarity criteria followed for alignment [15].

Thus, as shown in figure 1, the epitope recognized by the CB-EGF1 MAb comprises aminoacids S9, H10, Y13, K28, E40 and R41.

This conformational epitope includes the Y13 and R41 residues, essential for the EGF-EGFR interaction (Figure 2) and defining the neutralizing activity of the MAb as previously characterized in EGF-EGFR binding inhibition assays [11]. Peptides isolated from the pVIII-9CaaC cyclic peptide library resembled the consensus SKFDR sequence identified from the linear peptide library.

All peptides were mimicking the EGF region interacting with the βchain of the EGFR, as determined by molecular modeling with the WHATIF software [16], bearing aromatic (Y/F) and charged (K/R) neighboring residues.

The most frequent peptides for both libraries (F22-54: CPAKFSPSVC and F22-55: AKFNDYWRW) were synthesized and evaluated in a direct binding ELISA. They were weakly recognized by the MAb, as shown in figure 3. A possible explanation involves the hard-to-achieve peptide coupling to im-munoreactive surfaces, due to their low molecular size [17]. Pre-treating plates

Table 2. Peptide sequences obtained after rounds of selection with libraries pVIII-9aa (linear peptides) and pVIII-9CaaC (cyclic peptides).

Library	
pVIII9aa	pVIII9CaaC
---AKFNDYWRW-—-	CPPAKFSPSVC
---SKFDAPRL---P	CHKFDQLQRPC
---LKFNRPPG-L--	CSSLDTRFWAC
--QRSFDGP-P----	CSLARSKFSAC
EHALKFGP------P	CTAHERKFVAC
-SLAKFGPPP-----	CPPAKFSQAC
---SKFDAPRLP---	CTHLSADSTSC
----KFGPKTVLP--	CANRFDEKALC
Cons---SKFDR-------	CRRWSASTKAC

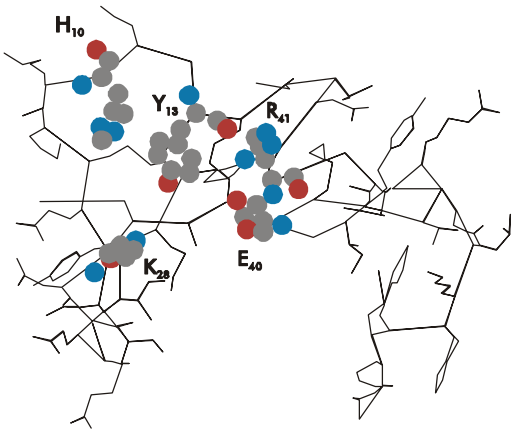

Figure 1. Site recognized by the CB-EGF1 monoclonal antibody, according to EGF crystallography data analyzed with the Web lab viewer lite software [16]. Aminoacids represented in single letter code.

with glutaraldehyde guarantees peptide attachment, but generates multimeric peptide complexes that interfere with antibody recognition [18], and reacts with residues critical for MAb binding such as K/R and Y/F. In spite of the poor differences observed between positive peptide samples and the negative control, duplicates ranged within well-defi-ned intervals, with variation resulting from binding reactions but not due to sample variability.

These results were confirmed by a competition ELISA, also determining the capacity of peptides F22-54 and F22-55 to inhibit the EGF-MAb interaction. For this purpose, CB-EGF1 samples were incubated with each peptide before to incubation with EGF, in polyestirene plates. As shown in figure 4, peptides F22-54 and F22-55 evidenced 45% and 30% binding inhibition, respectively. The difference was statistically significant for the F22-54 peptide, according to Duncan's Multiple Range Test ($p<0.05$). Differences in sequence, conformation and molecular species present within peptide preparations would determine the results observed.

To analyze the possible effect of both peptides on the EGF-EGFR system, 3T3 A31 cells (ECACC) depending on EGF for growth, were incubated with four 1:2 serial dilutions of each peptide (100 µg to 12.5 µg) in the presence or absence of 1.88 IU/mL

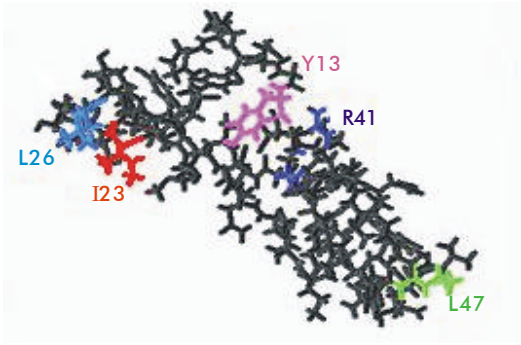

Figure 2. Structural modeling of the EGF surface interacting with the EGFR. Binding residues are highlighted.

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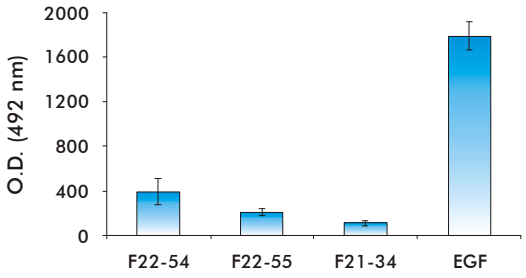

Figure 3. Immunochemical recognition of synthetic peptides F22-54 and F22-55 by the CB-EGF1 MAb. EGF and peptide F21-34 were used as positive and negative controls, respectively.

EGF, or the culture medium as the negative control. After 24 h of incubation, O.D. values at 578 nm indicated the lack of activity in this system for both peptides, as shown in figure 5. Results obtained with or without EGF were similar to experimental control values.

According to the WHATIF data analyzed, peptides F22-54 and F22-55 are able to bind to the β chain of the EGFR. However, they do not interact with the α chain, failing to activate the receptor. The interaction of EGF with the α chain in the EGFR promotes conformational changes essential to bring EGFR domains I and III close enough to promote activation [19].

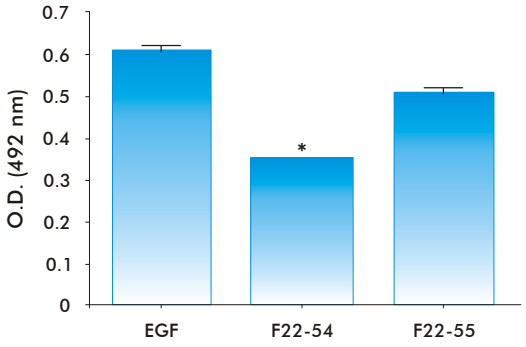

Figure 4. Inhibition of CB-EGF1 bingding to EGF by peptides F22-54 and F22-55. *p< 0.05 compared to EGF (Duncan´s multiple range test).

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It is known that peptides selected through the use of phage display technology usually show less potency when synthesized as individual compounds [20, 21]. Two main reasons account for this behavior: a) insufficient conformational stability of these small molecules for a strong interaction with the target molecule (in the phage context such stability is provided by the fusion to the coat protein); and b) phage particles carry multiple copies of the fusion protein; this multivalency is responsible for an avidity higher than that achieved with synthetic peptides.

Binding and multivalency of synthetic compounds could be increased by strategies involving the clustering of multiple copies through conjugational coupling to molecular scaffolds like proteins, liposomes, polymers and others [22-24].

Once more, the phage display technology proves useful in selecting peptides and proteins capable of binding to a target molecule. Peptides selected in this work can be used to study mechanisms of molecular recognition, molecular mimicry of ligands, or to be considered for designing compounds relevant in the-rapy or diagnostics.

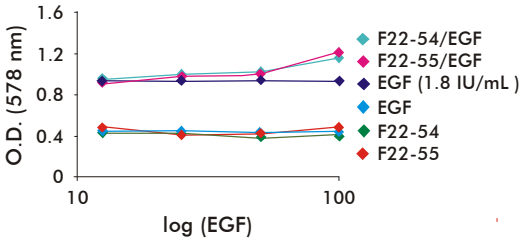

Figure 5: Cell proliferation assay for F22-54 and F22-55 peptides with or without medium.

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