

Expression of Intracellular Domain of Epidermal Growth Factor Receptor and Generation of Its Monoclonal Antibody

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To prepare monoclonal antibody specific to epidermal growth factor receptor (EGFR) intracellular domain, its gene was amplified from total RNA of A431 cell by RT-PCR. Then the gene was cloned into prokaryotic vector pET30a(+). The recombinant plasmid was transformed into *E. coli* BL21 (DE3) strain for protein expression. Recombinant protein was induced with IPTG and purified using Ni²⁺-NTA agarose. Then the anti-EGFR monoclonal antibody (mAb) was prepared with classical hybridoma technique. Positive clones were selected using indirect enzyme-linked immunosorbent assay (ELISA). Totally 4 hybridoma clones were obtained and these mAbs were IgG1 (3 clones) and IgG2a (1 clone), respectively. Their light chains were all kappa chains. Western blotting analysis and confocal immunofluorescence assays demonstrated that mAbs could specifically recognize EGFR expressing on A431 carcinoma cell line. The mAbs will be useful in the study of EGFR-mediated signal transduction. *Cellular & Molecular Immunology*. 2004;1(2):137-141.

Key Words: EGFR, prokaryotic expression, monoclonal antibody, intracellular domain

Introduction

The epidermal growth factor receptor (EGFR) is a transmembrane protein with intrinsic tyrosine kinase activity, which has also been referred to as HER1 (human EGF receptor) and c-erbB-1. Structurally, EGFR is a 170 kD glycoprotein that contains an extracellular ligand-binding domain, a transmembrane region and an intracellular domain with TK activity (1-3). The receptor is activated by binding to at least five distinct ligands including EGF and transforming growth factor- β (TGF- β). Upon ligand binding, the EGFR undergoes autophosphorylation and initiates multiple intracellular signaling cascades. It plays an important role in the regulation of cell growth and differentiation (4-6).

EGFR and its ligands are involved in the cell growth of over 70% of all cancer cells. EGFR overexpression or dysregulation may alter intracellular signaling pathways and EGFR has been implicated in several pathways that affect tumor cell survival and apoptosis, proliferation and invasiveness. EGFR has been believed to be a therapeutic

target for cancer treatment (7, 8). Full elucidation of the mechanisms of behavior of both wild-type and oncogenic mutants of the EGFR should help with the design of new molecules to antagonize cancer.

In order to have EGFR-specific mAb and investigate EGFR-mediated signals, we cloned the truncated intracellular domain of EGFR and constructed the plasmid of pET30a(+)-EGFR and its recombinant protein was expressed in prokaryotic expression system. The recombinant protein was induced by IPTG and purified with Ni²⁺-NTA agarose. Mouse monoclonal antibodies were produced by immunization with the purified recombinant protein. These mAbs can specifically react with the EGFR expressing on the A431 carcinoma cell line. Furthermore, when recombinant EGF was used to stimulate A431 cell line, EGFR translocated from cell membrane to cytosol of the cells. Our mAb was able to bind to EGFR on the cell membrane and in the cytosol of the cells. Our data demonstrated that the EGFR-specific mAb would be helpful to detect EGFR expression and to study its regulatory role in a diverse set of cellular processes.

Materials and Methods

Materials

Expression plasmid pET30a(+), *E. coli* DH5 α and BL21 (DE3) strains were purchased from Novagen. Restriction Enzyme was purchased from Takara. T4 DNA ligase was purchased from SinoAmerican Biotechnology Company. Trizol reagent and MMLV Reverse Transcriptase were

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Received for publication Mar 22, 2004. Accepted for publication Apr 12, 2004.

Abbreviations: EGFR, epidermal growth factor receptor; RT-PCR, reverse transcription-polymerase chain reaction; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; IPTG, isopropyl- β -D-thiogalactopyranoside; PMSF, phenylmethylsulfonylfluoride; mAb, monoclonal antibody.

from Gibco BRL Company. IPTG, PMSF, and Taq DNA polymerase were from Promega Company. EGF was purchased from Calbiochem Company. CFA was from Sigma Company.

RT-PCR

Total RNA was isolated from cultured A431 cells using Trizol reagent according to the manufacturer and reverse-transcribed using oligo dT primers with the MMLV reverse transcriptase. The PCR product of EGFR gene was ligated to pGEM-T vector following the manufacturer's instructions. The positive clones were selected and confirmed by DNA sequencing analysis.

Protein expression and purification

All DNA manipulation and bacterial transformation were based upon the methods described by Sambrook et al. (9). The recombinant plasmid pGEM-T-EGFR was digested with Sall and BamHI and cloned into pET30a(+) (Novagen), and then transformed into *E. coli* BL21 (DE3). The transformed *E. coli* strain was cultured at 30°C in 500ml LB medium with kanamycin until the cell density reached OD₆₀₀≈0.6. Then IPTG was added to a final concentration of 0.5 mM. After continuing culture for 4h, the cells were collected. Then the cells were resuspended in 8ml of lysis buffer and sonicated 30s with 30s pause at 120W on ice. The lysate was centrifuged at 6,500 rpm at 4°C for 10 min. The supernatants were collected and saved on ice for purification using Ni²⁺-NTA agarose following the manufacturer's protocol.

SDS-PAGE

Samples for SDS-PAGE (performed according to standard procedures) were centrifuged, and cell pellets were suspended in loading buffer and heated to 100°C for 5 min. Debris was removed by centrifugation and the supernatants were analyzed by SDS-PAGE on 10% gels stained with Coomassie Brilliant Blue R250.

Western blotting

Protein samples (10μl) were electrophoresized with 10% SDS-PAGE and then were transferred onto the PVDF membrane with Bio-Rad equipments. The membrane was block with 3% BSA for 1h. After incubating with mouse anti-His-tag antibody (Qiagen) for 1h, the membrane was stained with goat anti-mouse IgG-HRP-conjugated antibody (Sigma) for 1h. Then the blotting signals were developed using DAB Stock Stain Kit (Sino-American Biotechnology Co.).

Generation of monoclonal antibody against EGFR

Female Balb/c mice were immunized three times with the purified recombinant protein. Spleen cells from the immunized mouse were fused with SP2/0 murine myeloma cells through standard procedures. Culture supernatants of the hybridoma were screened using indirect ELISA. After repeated screening and cloning with limiting dilution, four hybridoma clones were obtained. The mAb isotyping was carried out by using mouse sub-isotyping panel (Bio-Rad).

Cell proliferation in response to EGF stimulation

Triplicate wells of 0.2 ml of A431 cells were incubated in

96-well tissue culture plates (1 × 10⁴ cells/well) for 24h, and then starved for another 24h. The cultures were incubated with or without EGF (0.01 nM or 1 nM). After 17h, the cultures were pulsed with [³H]-thymidine (0.5 μCi/20 μl/well) for an additional 2h. The cells were harvested and counted using standard liquid scintillation.

Confocal immunofluorescence analysis of the EGFR expression on A431 cell

A431 cells were cultured in RDF medium supplemented with 10% bovine serum and incubated at 37°C in a 5% CO₂ atmosphere. Cells grown on glass coverslips were starved for 24h and then with or without EGF(100 ng/ml) stimulating for 30 min. Then cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 (cytoperm/fix) for 15 min. After removal of the cytoperm/fix and washing with PBS, the samples were blocked with BSA for 15 min. Next the cells were incubated with monoclonal mouse anti-EGFR antibody (1:100) or mouse normal serum (1:100) at room temperature for 30 min. After washing with cytoperm/washing and PBS for 3 times respectively, the cells were incubated with donkey anti-mouse IgG conjugated with FITC (1:50) (Roche) for 30 min. Washing again, the cells were post-fixed with cytoperm/fix for 15 min and mounted with 50% glycerol then were examined by confocal microscopy (Radiance2100, Bio-Rad).

Results

Construction of the recombinant expression plasmid pET30a(+)-EGFR

The full length of EGFR gene was initially amplified from total RNA of A431 cell by RT-PCR and was cloned into EGFR-pGEM-T plasmid (Figure 1). Then the gene of truncated intracellular domain was inserted into prokaryotic expression vector (pET30a(+)) with BamHI and Sall. When pET30a(+)-EGFR plasmid was digested with Kpn I and Hind III, the constructed target gene (1kb) was notable in the gel (Figure 2). The inserted gene was confirmed by sequencing analysis (data not shown).

Expression and purification of recombinant protein

The recombinant expression plasmid (pET30a(+)-EGFR)

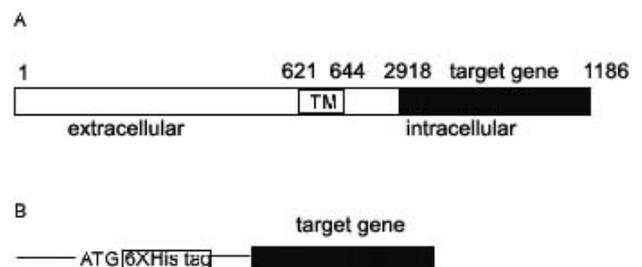


Figure 1. Structure of the EGFR gene and the construction of the recombinant expression plasmid. (A) Schematic representation of domains of the epidermal growth factor receptor sequence. TM: transmembrane domain, the numbers shown represent amino acid position. (B) Structure of recombinant plasmid pET30a(+)-EGFR.

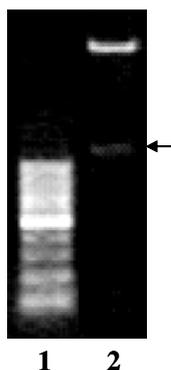


Figure 2. Analysis of the plasmid (pET30a(+)-EGFR) by double digestion. Lane 1, 100bp DNA marker; Lane 2, The pET30a(+)-EGFR plasmid was digested with Kpn I and Hind III. The two bands were generated. A large one is vector (5.4kb) and the small one was target gene of EGFR (1kb).

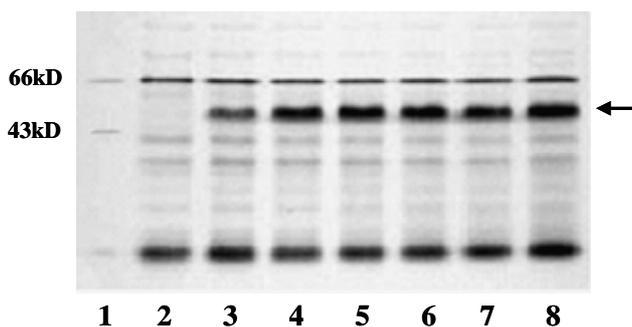


Figure 3. SDS-PAGE analysis of the expression of recombinant protein. Lane 1, Low weight standard protein marker; Lanes 2, *E. coli* BL-21(DE3) cells transformed with pET30a(+) control vector. Lane 3-8, *E. coli* BL-21(DE3) cells transformed with pET30a(+)-EGFR were induced by 0.5 mM IPTG. Aliquots were taken at hour intervals, analyzed on a 10% SDS gel and stained with Coomassie Brilliant Blue.

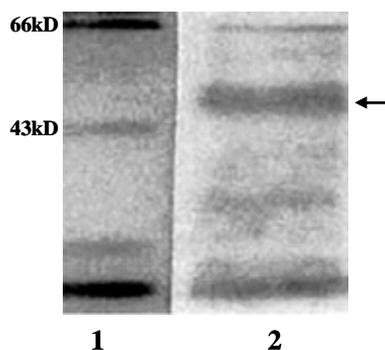


Figure 4. Western blotting analysis of the recombinant protein. Lane 1, Low weight standard protein marker; Lane 2, *E. coli* BL-21(DE3) cells transformed with pET30a(+)-EGFR were induced with IPTG and the expressed protein was confirmed by anti-His Ab.

was transformed into *E. coli* BL21 (DE3); EGFR was then highly expressed after induced by IPTG. As expected, the target protein (45 kD) was expressed (Figure 3). In order to determine whether the fusion protein was soluble or in inclusion body, the cell pellet was resuspended and sonicated on ice in proper buffer. It was found that the recombinant protein was mainly in soluble form. The

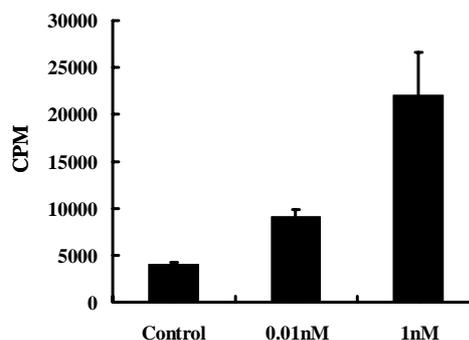


Figure 5. A431 cell proliferation assay. A431 cell line was stimulated with different concentrations of EGF and cell proliferation was measured with standard liquid scintillation as described in materials and methods.

Table 1. Titers of anti-EGFR monoclonal antibodies in supernatants* of hybridoma clone cultures and their isotypes.

Clone	Ab titer**(OD ₄₅₀ ±SD)	Ig subclass
23-7	2.087±0.193	IgG1,κ
224-7	1.956±0.145	IgG2a,κ
296-26	2.160±0.090	IgG1,κ
11-9	2.059±0.077	IgG1,κ

* The supernatant was from the culture of 1×10^6 hybridoma cells in 9ml medium for 3 days.

**Results of ELISA.

lysate supernatant was subjected to Ni²⁺-NTA agarose column for affinity chromatography purification. The purified protein was further confirmed by Western blotting using anti-His monoclonal antibody. The data showed that the truncated EGFR was expressed (Figure 4).

Four hybridoma clones were raised in EGFR protein immunized mouse

After three times immunization with EGFR protein in CFA, the spleen cells from immunized mouse were fused with SP2/0 cells. Through 3 times of cloning by limiting dilution, four hybridoma clones were obtained. The data showed that all the clones secreted higher titer of mAbs which were specific to EGFR (Table 1).

Cell proliferation in response to EGF stimulation

To ensure whether our working system was good, the response of A431 cells to EGF was measured. After serum starved, the A431 cells were stimulated with the different concentration of EGF. It was observed that A431 cells could proliferate in response to the stimulation of EGF and the cell proliferation was increased following the dose change of EGF (Figure 5).

Confocal immunofluorescence analysis of the EGFR expression on A431 cells

Since EGF-stimulated A431 cell was a good system to

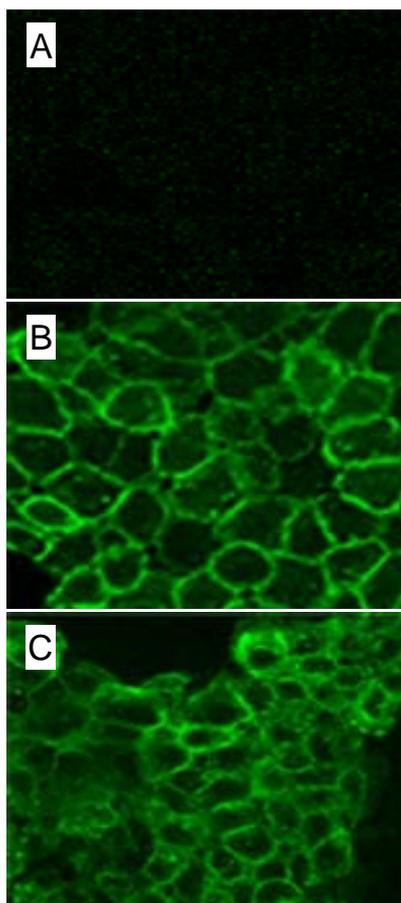


Figure 6. Confocal analysis of EGFR expression on A431 cells. The A431 cells were stimulated with or without EGF stimulation and EGFR was determined in the cell membrane and cytosol. (A) Without anti-EGFR antibody, only FITC-labeled anti-mouse IgG was used. (B) using anti-EGFR antibody as the primary antibody, without EGF stimuli. magnified $\times 100$. (C) using anti-EGFR antibody as the primary antibody, with EGF(100ng/mL) stimuli. magnified $\times 100$.

evaluate the function of EGFR, the specificity of the mAbs to EGFR was measured in the cells. To determine whether the raised monoclonal antibody was capable of recognizing the EGFR expressing on A431 cell lines, the confocal microscope technology was used to address the question. The A431 cell lines were incubated with the monoclonal antibodies, then stained with the donkey FITC conjugated anti-mouse IgG. We observed that without stimulation of EGF, green fluorescent was mainly located on the cell membrane, suggesting that the mAb could bind to EGFR on the cell membrane. It was also interesting to note that after stimulation with EGF, the EGFR could be detected in the cytosol of the cells, indicating that once the A431 is activated with EGF, the part of EGFR translocated from cell membrane to cytosol where it regulated cell function (Figure 6).

Discussion

The EGFR mediates multiple cellular functions, including

cell proliferation, migration, apoptosis and differentiation. EGFR signaling is tightly controlled in normal cells, but when EGFR is overexpressed, it promotes oncogene activation and alters the signaling pathways of the cells. EGFR is widely expressed on many cancer cells. EGFR represents a promising therapeutic target. The expression and function of EGFR play an important role in the growth of cancer cells.

It is well known that once the EGF binds to EGFR extracellular domains, the proportion of dimerized receptor is increased and subsequently the enzymatic activity of EGFR intracellular tyrosine kinase is enhanced greatly. The EGFR kinase catalyses the transfer of the γ -phosphate of bound ATP to the tyrosine residues of exogenous substrates and the C-terminal domains of the EGFR. After the induction of tyrosine phosphorylation, some signaling pathways appear to start with the recognition of the C-terminal phosphotyrosines by appropriate adaptor or signaling. The mechanism of the activation of the EGFR has been studied for many years, but it still remains to be determined.

In this study, we constructed prokaryotic expression plasmid pET30a(+)-EGFR, and the recombinant protein was expressed. After purifying the protein, we used it to immunize mice and generated the monoclonal antibodies successfully. The functional assay demonstrated that the mAbs could specifically recognize the EGFR expressing on A431 cell lines.

There was a paper indicated that EGFR, a trans-membrane receptor at the cell surface could somehow reappear as a transcription factor in the nucleus (10). Our observation found that EGFR was detectable in the cytosol of EGF stimulated A431 cells, suggesting that the EGFR translocates from cell membrane to the cytosol. Our data indicated that the translocation of EGFR might play an important role in the function of cells. The raised monoclonal antibody will be useful to study EGFR-mediated signal pathway.

Acknowledgement

This research work was supported by Science and Technology Commission of Shanghai Municipality (03JC14085), grant of National Natural Science Foundation # 30170888 and the grant from E-institutes of Shanghai Universities Immunology Division.

References

1. Carpenter G. Receptors for epidermal growth factor and other polypeptide mitogens. *Annu Rev Biochem.* 1987;56:881-914.
2. Hunter T, Cooper JA. Protein-tyrosine kinases. *Annu Rev Biochem.* 1985;54:897-930.
3. Wells A. EGF receptor. *Int J Biochem Cell Biol.* 1999;31:637-643.
4. Harris RC, Chung E, Coffey RJ. EGF receptor ligands. *Exp Cell Res.* 2003;284:2-13.
5. Bogdan S, Klambt C. Epidermal growth factor receptor signaling. *Curr Biol.* 2001;11:R292-295.
6. Hackel PO, Zwick E, Prenzel N, Ullrich A. Epidermal growth factor receptors: critical mediators of multiple receptor pathways. *Curr Opin Cell Biol.* 1999;11:184-189.

7. Foon KA, Yang XD, Weiner LM, et al. Preclinical and clinical evaluations of ABX-EGF, a fully human anti-epidermal growth factor receptor antibody. *Int J Radiat Oncol Biol Phys.* 2004;58:984-990.
8. Todderud G, Carpenter G. Epidermal growth factor: the receptor and its function. *Biofactors.* 1989;2:11-15.
9. Fry DW. Inhibition of the epidermal growth factor receptor family of tyrosine kinases as an approach to cancer chemotherapy: progression from reversible to irreversible inhibitors. *Pharmacol Ther.* 1999;82:207-218.
10. Cohen RB. Epidermal growth factor receptor as a therapeutic target in colorectal cancer. *Clin Colorectal Cancer.* 2003;2:246-251.
11. Sambrook J, Russell DW. *Molecular Cloning: A Laboratory Manual.* (third ed.). New York, NY: Cold Spring Harbor Laboratory Publishing; 2001.
12. Lin Shiaw-Yih, Makino K, Xia WY, et al. Nuclear localization of EGF receptor and its potential new role as a transcription factor. *Nat Cell Biol.* 2001;3:802-808.