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Identity of BCA200 and c-erbB-2 Indicated by Reactivity of Monoclonal Antibodies with Recombinant c-erbB-2

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Abstract

BCA200 has been described as a 200,000 Mr monomeric cell surface glycoprotein associated with human breast cancer. Since the physical properties and cellular distribution of BCA200 resemble those of c-erbB-2, antibodies to BCA200 were tested for the ability to bind a recombinant protein containing the c-erbB-2 extracellular domain (erbB-2 ECD). Three antibodies to distinct epitopes of BCA200 reacted with erbB-2 ECD but not with a control protein expressed in a similar baculovirus lysate. Control myeloma proteins and antibodies to four other antigens did not react with erbB-2 ECD. A protein with the expected molecular weight for erbB-2 ECD was also immunoprecipitated by anti-BCA200 antibody 520C9. We conclude that BCA200 is another synonym for c-erbB-2.

Introduction

C-erbB-2 or HER-2 is a human proto-oncogene homologous to the rat neu oncogene and related in sequence to human erbB-1 or epidermal growth factor receptor (Coussens et al., 1985; Bargmann et al., 1986; Yamamoto et al., 1986). It encodes a 185-190,000 dalton protein product with extracellular, transmembrane and tyrosine kinase domains (Akiyama et al., 1986; Yamamoto et al., 1986). Normal c-erbB-2 product has transforming activity when over-expressed, as do several mutant gene products with amino acid substitutions in the transmembrane region (Di Fiore et al., 1987; Slamon et al., 1989). Gene amplification and protein level over-expression of c-erbB-2 have been observed in a wide variety of human adenocarcinomas and correlated with metastasis and poor prognosis (Slamon et al., 1987, 1989; Berger et al., 1988; Zhou et al., 1987).

Recently, one of us described BCA200, an approximately 200,000 M_r glycoprotein recognized by a number of mouse monoclonal antibodies raised against human breast cancer (Ring et al., 1989). In discussing the relationship of BCA200 to known tumor-associated antigens, we noted that a polyclonal rabbit serum against c-erbB-2 did not capture antigen recognized by our BCA200 antibodies. However, many properties of BCA200 (Mr, monomeric glycoprotein structure, expression levels on particular cell lines, tumor types and normal human tissues) resembled those reported for c-erbB-2, leading us to question the negative result of the antigen capture experiment. (We were concerned that the polyclonal capture antibody was not affinity purified, and may actually have captured very little c-erbB-2). We have now carried out experiments showing that antibodies to three distinct epitopes on BCA200 react with a recombinant protein containing the extracellular domain of c-erbB-2.

Materials and Methods

PCR subcloning of the c-erbB-2 extracellular domain (erbB-2 ECD). cDNA template was prepared by reverse transcription of mRNA from tumor T882 (a gift of Dennis Slamon, UCLA). The cDNA was synthesized by using murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) according to the manufacturer's protocol. The 20 µl reaction mixture contained the enzyme buffer as supplied, 0.5 µg RNA, 10 units of RNasin (Promega Biotec, Madison, WI), 10 pmol of 3' PCR primer (CAGTCTAGATTTCAGGATCCGCATCTGCGC), 1 mM (each) deoxynucleoside triphosphates, and 100 units of reverse transcriptase. The reaction mixture was incubated for 30 min at 37 °C. For amplification (Mullis & Faloona, 1987; Saiki et al., 1985) 4 µl of the reaction mixture was then diluted into 100 µl PCR buffer (10 mM Tris pH 8.4, 2.5 mM $MgCl_2$, 50 mM KCl, 100 µg/ml BSA) containing 100 µM dNTP's, 1 pmol of each primer and 0.5 unit of thermostable DNA polymerase from *Thermus aquaticus* (*Taq* polymerase; Chien et al., 1976; Saiki et al., 1988). The reaction was started by denaturing the RNA cDNA hybrid by heat (95 °C) for 1 min, annealing the primers for 1 min at 55 °C, and then extending the primers at 72 °C for 3 min. This cycle was repeated 25 times using a programmable heat block designed and manufactured by Cetus Corporation (Emeryville, CA). After the final cycle, the temperature was held at 72 °C for 10 min to allow reannealing of the amplified products and then was chilled. PCR amplification of the 5' half erbB-2 ECD coding sequence was directed by the 5' primer GCACCATGGAGCTGGCGGCC and the 3' primer GCACTTCTCACACCGCTGTGTTCC. Amplification

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of the 3' half erbB-2 ECD coding sequence was directed by the 5' primer CGGACGTGGGATCCTGCACCCTCGT-CTGC and the 3' primer CCTGGTACCTTATGTTTCAG-GTTCAGGAGGAGGTGTGCCGGACGTCAGAGGGCT GGCTCTCTGCTCG. (Underlined nucleotides encode the KT3 epitope.) Products obtained after amplification were inserted between the NcoI and EcoRI sites of baculovirus transfer vector pAcC4 (Luckow and Summers, 1988) to produce the transfer vector pAcNU2. Sequencing of the cloned PCR product revealed three alterations in coding sequence (F 173 to L, L 608 to P, I 613 to V) compared to the previously published sequence (Coussens et al., 1985). These three changes are likely to be artifacts of PCR amplification.

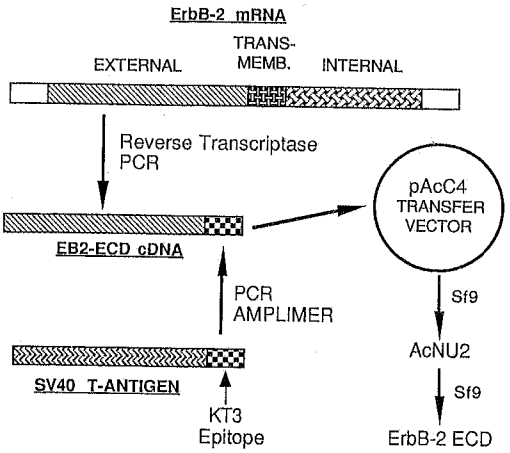

Figure 1. Construction of the cloned extracellular domain of c-erbB-2 (erbB-2 ECD).

Expression of erbB-2 ECD in insect cells. Sequence encoding erbB-2 ECD was recombined into the Autographa californica baculovirus (AcNPV) via the transfer vector pAcNU2. To generate recombinant virus, 2 µg of transfer vector was co-transfected with 1 µg of wild type viral DNA into Sf9 cells as described (Summers and Smith, 1987). Recombinant virus (occlusion-negative) was isolated from the transfection supernatant by plaque purification (Smith et al., 1983). To produce recombinant proteins, Sf9 cells were infected with 5-10 PFU of recombinant virus per cell. Suspension cultures were grown in a stirred flask containing protein-free medium (Maiorella et al., 1988). The cells were infected at 1-1.5 x 10^6 cells per ml and were harvested at 48 hr post-infection. The expression level of recombinant protein was approximately 0.4 mg/l of Sf9 culture.

Monoclonal antibodies. Murine monoclonal antibodies 106A10 (IgG_1), 113F1 (IgG_3), 454A12 (IgG_1), 454C11 (IgG_2a), 520C9 (IgG_1) and 758G5 (IgG_1) resulted from immunizations with human breast carcinoma cell lines or membrane preparations, and were purified from mouse ascites as previously described (Frankel et al., 1985). Murine hybridoma 3G8 (IgG_1) (Unkeless, 1979) was obtained from Dr. Jay Unkeless and antibody was purified from mouse ascites by size exclusion and anion exchange chromatography. Murine hybridoma KT3 (IgG_1) (McArthur and Walter, 1984) was obtained from Gernot Walter and KT3 antibody was purified by anion exchange. MOPC21 and RPC5 myeloma proteins were obtained from Zymed Laboratories, South San Francisco, CA; horseradish peroxidase conjugates of monoclonal antibodies were also prepared by Zymed.

Immunoprecipitation of erbB-2 ECD by KT3 and 520C9 monoclonal antibodies. KT3-Sepharose was prepared by absorbing KT3 monoclonal antibody (MacArthur and Walter, 1984) to protein-G Sepharose (5-8 mg KT3 per ml resin) and then cross-linking with dimethyl pimelimidate (Schneider et al., 1982). Supernatants from AcNU2 or control-infected Sf9 cells were concentrated 10-fold and diafiltered 10-fold into PBS using 30 kilodalton Centri-prep (Amicon) diafilters. They were then incubated with 8 µl KT3 Sepharose and 5 µg 520C9 or MOPC-175 myeloma protein or no addition for 1 hr at room temperature and 1 hr at 4 °C. 8 µl protein-G Sepharose (Pharmacia) was added after the first hour. The Sepharose beads were washed 4 times with 1 ml 20 mM Tris pH 8, 150 mM NaCl, 0.5% NP40 and extracted with SDS PAGE sample buffer. Extracted proteins were resolved by SDS polyacrylamide (8%) gel electrophoresis and visualized by Coomassie blue staining.

ELISA. A polyvinyl chloride microtiter plate was coated overnight with KT3 antibody at 10 µg/ml in 50 mM $NaHCO_3$ pH 9.5. After three rinses in PBS, wells were incubated 1 hour in 50 µl PBS or SF9 cell supernatant containing recombinant c-erbB-2 or M-CSF protein. After another three rinses in blocking solution (10% heat inactivated fetal bovine serum, 2% normal mouse serum, 1% nonfat dry milk, 1% bovine serum albumin in PBS), wells were incubated 1 hour in 50 µl blocking solution containing antibody-peroxidase conjugates at 1 µg/ml. After four final PBS rinses, wells were developed with tetramethylbenzidine substrate and absorbances were read at 450 nm as described (Sheldon et al., 1986). All operations were conducted at room temperature.

Results and Discussion

cDNA encoding the extracellular domain of c-erbB-2 (erbB-2 ECD) was synthesized from tumor-derived mRNA by polymerase chain reaction (PCR; Mullis & Faloona, 1987; Saiki et al., 1985) and cloned into a baculovirus transfer vector (pAcC4; Luckow and Summers, 1988). At the 3' end of this c-erbB-2 fragment, DNA encoding the late T antigen epitope (TPPPEPET or "Tag peptide") was added (Figure 1). This epitope is recognized by the monoclonal antibody KT3 (MacArthur and Walter, 1984). erbB-2 ECD was expressed in Sf9 insect cells using the AcNPV baculovirus expression system (Smith et al., 1983; Summers and Smith, 1987; Luckow and Summers, 1988). A 90 Kd protein was specifically immunoprecipitated by KT3 from supernatants from Sf9 cells infected with erbB-2 ECD encoding virus (AcNU2) but not from control-infected cells (Figure 2). The identity of this protein was confirmed by N-terminal sequence analysis. (Note that Figure 2 shows Coomassie blue staining of precipitating antibodies as well as immunoprecipitated antigens.)

Figure 3 shows the reactivity of various antibody probes with erbB-2 ECD. Microtiter wells were coated with antibody KT3, and subsequently incubated with AcNU2 supernatant containing erbB-2 ECD or with a similar infected Sf9 supernatant containing recombinant M-CSF. Antibodies 454C11, 520C9 and 758G5 recognize different antigenic determinants on BCA200 (Ring et al., 1989) and MOPC21 and RPC5 are isotype-matched myeloma proteins without known binding specificities. 106A10 and 113F1 respectively immunoprecipitate a 55,000 M_r glycoprotein and a 40/60/100/200,000 Mr set of glycoproteins (D. Ring, unpublished data); 454A12 recognizes human transferrin receptor

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