Monoclonal Antibodies against the Extracellular Domain of the erbB-2 Receptor Function as Partial Ligand Agonists*

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In this paper we describe the isolation and characterization of four monoclonal antibodies (FRP5, FSP16, FWP51, and FSP77) which specifically recognize the human erbB-2 protein. All of the antibodies recognize epitopes on the extracellular domain of the receptor protein. FRP5 and FSP16 compete with one another for binding while FWP51 and FSP77 each recognize a different epitope. The effects of the antibodies on the erbB-2 receptor protein have been analyzed. Two different erbB-2-expressing cell lines, SKBR3 breast tumor cells and HC11 R111 cells, were examined. The SKBR3 cells express approximately 1 x 10⁶ molecules of the erbB-2 protein/cell; HC11 R111 cells, a clone of mouse mammary epithelial cells derived by transfection of a human erbB-2 expression plasmid, contain 10-fold less erbB-2 protein than the SKBR3 cells. Treatment of the two cell lines with FRP5, FSP16, and FWP51 led to a rapid increase in the phosphotyrosine content of the erbB-2 protein. Three of the antibodies, FRP5, FSP16, and FSP77, stimulated the turnover of the erbB-2 protein. Binding of the antibodies did not stimulate DNA synthesis in HC11 R111 cells. Thus, the erbB-2-specific monoclonal antibodies behave as partial ligand agonists. The antibodies were examined for their effects upon the growth of SKBR3 and HC11 R111 cells. The growth of SKBR3 cells was inhibited by 90% following long term treatment of the cells with FSP77.

Multiple genetic alterations have been detected in the DNA of tumor cells (1). Technological advances have made it possible to analyze these mutations at the molecular level. It has become clear that in particular tumor types such as colorectal (2) or breast cancers (3) there are consistent patterns of DNA alterations. This suggests that the mutated genes are involved in the development of the tumor. The erbB-2 is a gene which appears to be important in breast and ovarian tumor development. Amplification of the erbB-2 gene, leading to over-expression of the protein, has been observed in a high percentage of human breast and ovarian tumor cells (4-8). The erbB-2 protein is a member of the receptor tyrosine kinase family and is most closely related to the epidermal growth factor (EGF)* receptor (9). The erbB-2 protein has been the object of intensive study following the discovery of its involvement in cancer. Clinical studies have determined its role as a prognostic indicator in breast and ovarian cancers (8, 10). Molecular biological studies aimed at understanding its function in normal and transformed cells have also been undertaken (11, 12). Since the erbB-2 protein has an extracellular domain and is expressed at high levels in some tumors it is also a potential target for anti-tumor agents. As a first step in producing such reagents, we have isolated monoclonal antibodies (mAbs) which recognize the human erbB-2 protein. In this paper we describe the characterization of four mAbs which specifically bind to the extracellular domain of the erbB-2 protein. These antibodies act as partial ligand agonists. Following binding of the antibodies to the erbB-2 protein on intact cells, there is an increase in the phosphotyrosine content of the receptor and of other cellular proteins. Three of the antibodies cause an increase in the degradation of the erbB-2 protein. In addition, one antibody has strong growth inhibitory effects upon the SKBR3 breast tumor cell line which expresses high levels of the erbB-2 protein.

MATERIALS AND METHODS

Cell Culture—The SKBR3 human breast tumor cell line and the A431 human epidermoid carcinoma cell line were maintained in Dulbecco's modified Eagle's medium containing 8% Nu-serum (Collaborative Research). HC11 mouse mammary epithelial cells (13) and clone R111, a transfected HC11 cell clone expressing the human erbB-2 protein (14) were grown in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (FCS), 5 μg/ml bovine insulin (Sigma), 10 ng/ml murine EGF (Fluka), and 2 mM glutamine. Hybridoma cells were maintained in RPMI-1640 containing 20% heat-inactivated FCS, 4 mM glutamine, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol. In one experiment the SKBR3 cells were treated 8 h with the antibiotic tunicamycin (Boehringer) at a concentration of 5 μg/ml of growth medium.

Preparation of Cells for Immunization—Female BALB/c mice were immunized with intact SKBR3 human breast tumor cells. These cells have amplified copies of the erbB-2 gene (15) and express approximately 1 x 10⁶ molecules of the erbB-2 protein/cell. The cells were washed once with phosphate-buffered saline (PBS) and detached from the tissue culture plate at 37 °C in PBS containing 25 mM EDTA and 0.15 M NaCl. After low speed centrifugation, the cells were suspended in cold PBS and mixed 1:1 (v/v) with Freund's complete adjuvant. 2 x 10⁶ cells were injected either intraperitoneally or subcutaneously into BALB/c mice. The injections were repeated intraperitoneally several times over a period of 3-4 months. Three days before fusion a final injection of SKBR3 cells was given intraperitoneally.

Fusion and Screening Procedure—Mice were sacrificed and their spleen cells were fused according to standard procedures (16) with the mouse myeloma cell line P3X63-Ag8.653 at a ratio of 5:1. Polyethylene glycol 4000 was used as the fusing agent, and the cells were plated on petri plates and microplugs at a density of 1 x 10⁶ cells/well of a 24-well tissue culture dish in hypoxanthine/aminopterin/thymidine selection medium.

The hybridoma cells were screened for the production of erbB-2-specific antibodies in two steps. Hybridoma supernatants were tested for their immunofluorescent staining of a clone of transfected HC11...
mouse mammary epithelial cells expressing the human erbB-2 protein (clone R111) (14). Nontransfected HC11 cells served as a control. Cells were grown 1–2 days on fibronectin-coated coverslips, rinsed in PBS, and fixed by 10-min treatment with 3% formaldehyde made in PBS. Hybridoma supernatants were added to the cells, and the coverslips were incubated 1–2 h at 4°C. Immunofluorescent staining was determined using fluorescein-linked sheep anti-mouse Ig (Amersham). The positive supernatants were tested for specific immunoprecipitation of the erbB-2 protein from cell lysates of SKBR3 cells as described below.

Stable antibody-producing clones were obtained by limiting dilution in 96-well plates in a pristane-primed environment. Four anti-erbB-2 antibodies were isolated: mAbs FRP5, FSP16, FWP51, and FSP77. The purified mAbs were dialyzed against PBS and stored at −70°C.

Antibody Isoype and Subclass Determination—ELISA assays to determine the isotype and light chain class of the mAbs were done using a Bio-Rad Mouse Type TM Sub Isotyping Kit, as suggested by the manufacturer’s protocol. mAbs FRP5, FWP51, and FSP77 have the IgG1 isotype, and MAB FSP10 has the IgG2b isotype. All four of the antibodies have a light chain.

Immunoprecipitation and Immunoblotting—SKBR3 cell lysates were prepared by adding 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Triton X-100, 150 mM NaCl, 1 mM paramethysulfonil fluoride (PMSF) to a 10-cm plate of cells for 10 min at 4°C, followed by centrifugation at 10,000 × g for 10 min, erbB-2-Specific mAbs were added to the cell extract and allowed to bind for 2 h at 4°C, then rabbit anti-mouse Ig (ICN Immunobiologicals) was added for 1 h. The immune complexes were collected by the addition of protein A-Sepharose (Pharmacia). In one experiment a phospholipase C-γ1-specific MAb (UBI) was incubated with SKBR3 cell extract, and the immune complexes were collected by the addition of protein G-Sepharose (Pharmacia). The proteins were released by boiling in sample buffer and were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinyliden difluoride membranes (Millipore Corp.) (17). The erbB-2 protein was detected using the 21N antiserum which was raised in rabbits against a peptide representing residues 1243–1255 of the human erbB-2 protein (18) followed by treatment of the filter with 125I-labeled protein A (Amersham).

Effect of the Anti-erbB-2 Antibodies on the Phosphotyrosine Content of the Protein—SKBR3 or HC11 clone R111 cells were grown 24 h in medium containing 1% FCS, then treated 15 min at 37°C with the four antibodies at a concentration of 5–50 μg/ml of culture medium which contained no additional FCS or growth factors. Cell lysates were prepared using lysis buffer containing 200 μM sodium-orthovanadate and total lysates were separated by 7.5% SDS-PAGE. An immune complex was performed in duplicate, using a specific MAb (UBI) was incubated with the four antibodies at the same concentration and varying amounts of unlabeled antibody. The incubation was carried out in 250 μl of RIA buffer (120 mM NaCl, 50 mM HEPES, pH 7.8, 1 mM EDTA, 2% bovine serum albumin) for 2 h at 4°C. The cells were washed five times with RIA buffer, solubilized in 0.5 ml of 1% Triton X-100, 10% glycerol, 20 mM HEPES, pH 7.4, for 30 min at room temperature, and the specifically bound radioactivity was quantitated in a gamma counter. The results are an average from duplicate samples.

Tritiated Thymidine Incorporation Assay—1 × 10⁵ R111 cells were seeded/3.5-cm well of a multiwell tissue culture dish. When the cells reached 80% confluence the medium was replaced with one containing 1% FCS. One day later the cells were stimulated for 22 h with 10 mg/ml EGF or with 10 μg/ml of each MAb. [3H]Thymidine, at a concentration of 1 μCi/ml, was included for the final 4 h. The incorporation of [3H]thymidine into DNA was measured by washing the cultures with cold PBS, followed by a 5-min treatment with cold 0.5% trichloroacetic acid, then cold ethanol/ether 2:1. The plates were allowed to dry, then the cells were solubilized in 0.5 N NaOH, and the radioactivity was quantitated by liquid scintillation counting. The experimental points were done in duplicate.

Cell Proliferation Assay—1 × 10⁵ SKBR3 cells were seeded/3.5-cm well of a multiwell tissue culture dish in growth medium containing 1–10 μg/ml of each antibody. The medium was changed every 2 or 3 days, and at day 12 the cells were counted. The results are an average from duplicate wells.

RESULTS

Isolation of Monoclonal Antibodies Specific for the Human erbB-2 Protein—SKBR3 human breast tumor cells, which express high levels of the erbB-2 protein (15), were used as the immunogen for the production of erbB-2-specific mAbs. Intact cells were injected into BALB/c mice in order to optimize the chance of producing antibodies specific for the extracellular domain of the protein. Hybridomas were initially screened by an immunofluorescence assay using human erbB-2-transfected HC11 mouse epithelial cells, clone R111 (14), and control-untransfected HC11 cells. Those hybridomas whose supernatants stained the former cells positively and the latter negatively were cloned by limiting dilution, and their supernatants were screened by immunoprecipitating the erbB-2 protein from lysates of SKBR3 cells.

In order to test for specificity of the four mAbs, the following experiments were performed. SKBR3 human breast tumor cells and the human erbB-2-transfected mouse HC11 cells, clone R111, were labeled with [35S]methionine, and the erbB-2 protein was immunoprecipitated from lysates of the four mAbs. Fig. 1, A and C, shows that the four mAbs, FRP5, FSP16, FWP51, and FSP77 specifically immunoprecipitated the p185 erbB-2 protein from lysates of, respectively, SKBR3 cells and R111 cells. Fig. 1D shows that the mAbs do not recognize any proteins in the untransfected HC11 cells. The endogenous mouse erbB-2 protein can be immunoprecipitated from the HC11 cells by the 21N antiserum which recognizes the p185 protein from rodent cells (D, lane 1). These results show that the mAbs specifically recognize the human erbB-2 protein.

The A431 human epidermoid carcinoma cell line expresses approximately 2 × 10⁷ EGF receptors/cell (23) and only low levels of the erbB-2 protein (15). These cells were examined in order to show that the mAbs do not recognize the structurally related EGF receptor. The A431 cells were metabolically
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P

bles

p185

p170

FIG. 1. Immunoprecipitation of the erbB-2 protein from metabolically labeled cells with specific monoclonal antibodies. [35S]Methionine-labeled lysates from SKBR3 breast tumor cells (panel A), A431 epidermoid carcinoma cells (panel B), HC1 clone R111 cells (panel C), and HC11 cells (panel D) were immunoprecipitated with different antibodies and the immune complexes analyzed on SDS-PAGE as described under “Materials and Methods.” Panel A, lanes 2–5, immunoprecipitation with mAbs FRP5, FSP16, FWP51, and FSP77; lanes 1 and 6, immunoprecipitation with, respectively, rabbit anti-mouse IgG and a control unrelated mAb. Panel B, lanes 3–6, same as in panel A, lanes 2–5; lanes 1 and 2, immunoprecipitation with, respectively, EGF receptor-specific 15E antiserum and erbB-2-specific 21N antiserum. Panels C and D, lanes 3–6, same as in panel A, lanes 2–5; lanes 1 and 2, immunoprecipitation with, respectively, 21N serum and preimmune rabbit serum. The positions of the p185 erbB-2 receptor and the p170 EGF receptor are indicated.

labeled with [35S]methionine, and the erbB-2 protein was immunoprecipitated with each of the mAbs. Lanes 1 and 2 of Fig. 1B show the results of precipitating, respectively, the EGF receptor and the erbB-2 protein with specific sera. Lanes 3–6 show that each of the mAbs precipitates the p185 erbB-2 protein and does not recognize the p170 EGF receptor.

The mAbs Bind to the Extracellular Domain of the erbB-2 Receptor—In order to ascertain whether the mAbs recognize the extracellular domain of the erbB-2 protein, their binding was investigated by fluorescence-activated cell sorting. mAbs FRP5 and FSP16 were bound to intact SKBR3 cells. Fig. 2 shows an approximately 40-fold increase in the cellular fluorescence observed when mAbs FRP5 (panel A, solid line) and FSP16 (panel B, solid line) were incubated with SKBR3 cells, over that of a control mAb (panels A and B, dotted line). The results demonstrate that both mAbs bind to domains on the extracellular portion of the erbB-2 protein. mAbs FWP51 and FRP77 were shown by a similar experiment to bind to the extracellular portion of the erbB-2 protein.2

1. Harwerth and W. Wels, unpublished results.

FIG. 2. Fluorescence-activated cell sorter histogram of SKBR3 cells binding antibodies. In panel A the cells were allowed to react with mAb FRP5 (solid line) or a control unrelated mAb (dotted line); in panel B the cells were incubated with mAb FSP16 (solid line) or a control unrelated antibody (dotted line). Specifically bound antibody was detected with fluorescein-linked sheep antimouse serum, and the cells were analyzed for their fluorescence in a FACS

In order to characterize the nature of the epitopes recognized by the mAbs, SKBR3 cells were treated with the antibiotic tunicamycin, which prevents the addition of N-linked oligosaccharides to proteins (24). Fig. 3, lane 2, shows that the polyclonal 21N antiserum, which recognizes the carboxyl-terminal peptide of erbB-2, immunoprecipitates two proteins from tunicamycin-treated SKBR3 cells. The upper band co-migrates with the mature erbB-2 protein present in untreated cells, while the 170-kDa band represents unglycosylated protein. All four of the mAbs immunoprecipitate both forms of the erbB-2 protein from lysates of tunicamycin-treated cells (Fig. 3 lanes 4, 6, 8, and 10).

In order to determine whether the antibodies recognize the same or different determinants on the extracellular domain of the erbB-2 protein, cross-competition experiments using 125I-labeled mAbs were performed. The concentration of labeled mAb which saturated approximately 50% of the erbB-2-binding sites on the SKBR3 cells was determined, and the assays were performed in this range. SKBR3 cells were incubated with either 125I-labeled FRP5 or FSP77 in the presence of increasing concentrations of the four unlabeled antibodies. Fig. 4A shows that unlabeled mAb FRP5 and FSP16 were able to compete with labeled FRP5 for binding to the SKBR3 cells. Fig. 4B shows that only unlabeled mAb FSP77 was able to compete with labeled FSP77 in the binding assay. These results suggest that the four mAbs recognize at least three different antigenic determinants on the erbB-2 protein. mAbs FRP5 and FSP16 bind to similar regions on the extracellular
FIG. 3. Binding of specific mAbs to the erbB-2 protein from tunicamycin-treated SKBR3 cells. In lane 1 lysates from control SKBR3 cells were immunoprecipitated with 21N serum; in lanes 3, 5, 7, and 9 lysates were immunoprecipitated with, respectively, mAbs FRP5, FSP16, FWP51, and FSP77. In lane 2 lysates from tunicamycin-treated SKBR3 cells were immunoprecipitated with 21N serum; in lanes 4, 6, 8, and 10, lysates from tunicamycin-treated cells were immunoprecipitated with, respectively, mAbs FRP5, FSP16, FWP51, and FSP77. The precipitated proteins were electrophoresed, electrobotted, and the erbB-2 protein detected with 21N serum followed by 125I-protein A treatment of the filter. The positions of the glycosylated p185 erbB-2 protein and the unglycosylated p170 erbB-2 protein are indicated.

domain of the erbB-2 protein, while FWP51 and FSP77 bind to separate domains.

Binding of erbB-2-specific mAbs to Cells Leads to an Increase in the Phosphotyrosine Content of the Receptor and Other Cellular Proteins—Ligand binding to a receptor tyrosine kinase leads to its activation which can be measured by an increase in the phosphotyrosine content of the protein (reviewed in Ref. 25). Antibodies which mimic this characteristic of a ligand have been described for the EGF receptor (reviewed in Ref. 26), and they have been helpful in studying the biology of this receptor tyrosine kinase. Therefore, we have examined the effects which the erbB-2-specific mAbs have upon the phosphotyrosine content of the erbB-2 protein. SKBR3 and HC11 clone R111 cells were treated for 15 min at 37°C with 10 μg/ml of each mAb, and cell lysates were prepared in the presence of phosphatase inhibitors. Equal amounts of protein were examined by SDS-PAGE, and Western analyses were performed using a mAb specific for phosphotyrosine (19) and the 21N erbB-2-specific serum. The results obtained with SKBR3 cells and with R111 cells are presented in, respectively, Fig. 5, A and B, and C. Treatment of cells with the mAbs leads to an increase in the phosphotyrosine content of the receptor. The increase was quantitated with a phosphoimager. In SKBR3 cells, treatment with mAbs FRP5, FSP16, FWP51, and FSP77 led to an increase in the erbB-2 phosphotyrosine content of, respectively, 3.7-, 2.3-, 5.6-, and 1.8-fold (Fig. 5A, lanes 2–5). As a control, Fig. 5B shows that equal amounts of erbB-2 receptor were present in each of the extracts. Treatment of R111 cells with mAbs FRP5, FSP16, and FWP51 led to an increase in the erbB-2 phosphotyrosine content of, respectively, 3.0-, 3.3-, and 1.7-fold (Fig. 5C, lanes 3–5). The phosphotyrosine content of the erbB-2 receptor was not affected by treatment of R111 cells with mAb FSP77 (Fig. 5C, lane 6). Equal amounts of erbB-2 protein were present in all lanes. The results obtained with the two cell lines generally agree in that the mAbs FRP5, FSP16, and FWP51 have the strongest effect upon the erbB-2 phosphotyrosine content. mAb FSP77 had a weak effect upon the receptor in SKBR3 cells and no apparent effect in R111 cells. This may reflect the fact that R111 cells have approximately 10-fold less erbB-2 molecules/cell than do SKBR3 cells.

EGF addition to a variety of cells leads to an increase in the erbB-2 phosphotyrosine level (27–30). This phosphorylation most likely occurs via the formation of heterodimers between these two receptors (31). Fig. 5C, lane 1, shows the results obtained following EGF addition to R111 cells. Both the EGF receptor ( p170) and the erbB-2 receptor ( p185) display increased phosphotyrosine compared to the level seen in untreated cells (C, lane 2).

In order to test if the effect of the mAbs upon the erbB-2 phosphotyrosine content is dosage dependent, SKBR3 cells were treated 15 min at 37°C with 5 or 50 μg/ml of mAb FSP16, FWP51, or FSP77. Only treatment of the cells with mAb FWP51 results in a dosage-dependent increase in erbB-2 phosphotyrosine content (Fig. 6). Quantitation of the filter revealed a 2.8- and 4.2-fold increase in phosphotyrosine in cells treated with, respectively, 5 and 50 μg/ml FWP51. Treatment of the cells with both concentrations of FSP16 and FSP77 led to increases of, respectively, 2.0 and 1.7 in the erbB-2 phosphotyrosine content. These results agree well with those presented in Fig. 5A.

Activation of receptor tyrosine kinases leads to elevated levels of phosphotyrosine on cellular proteins which are putative substrates of the receptors. Fig. 7 shows that, in addi-
untreated. Equal amounts of cell lysates were analyzed on SDS-PAGE. The phosphotyrosine was detected with a specific mAb (19) and quantitated as described in Fig. 5. The positions of the p185 erbB-2 receptor and the p170 EGF receptor are indicated.

The cells were left untreated; in panels A and B, lane 1, the cells were left untreated; in panel C, lanes 1 and 2, cells were incubated, respectively, 10 min at 37 °C with 100 ng/ml EGF or left untreated. Equal amounts of cell lysates were analyzed on SDS-PAGE. The phosphotyrosine was detected with a specific mAb (19) (panels A and C), the erbB-2 was detected with 21N antiserum (panel B), and the filters were treated with 125I-protein A. The amount of radioactivity in each band was quantitated with a phosphoimager. The positions of the p185 erbB-2 receptor and the p170 EGF receptor are indicated.

The positions of the p185 erbB-2 protein and the p145 PLC-γ1 protein are indicated on the right. The arrows on the left show the positions of the prestained protein markers of 180, 116, and 84 kDAs.

FIG. 6. Concentration-dependent antibody induced tyrosine phosphorylation of the erbB-2 protein. SKBR3 cells were treated for 15 min at 37 °C with the indicated concentration of mAb. Equal amounts of cell lysates were analyzed on SDS-PAGE and the phosphotyrosine was detected and quantitated as described in Fig. 5. The position of the p185 erbB-2 protein is indicated.

Binding of Antibodies Accelerates the Turnover of the erbB-2 Protein in Intact Cells—Ligand-activated receptors are rapidly cleared from the cell surface via endocytosis through the coated pits (reviewed in Ref. 25). For the EGF receptor this can be measured by an increase in the turnover of the protein. Since binding of the antibodies enhances the in vivo phosphorylation of the erbB-2 protein, this suggests that they act as ligand agonists. We studied the next step in the activation pathway, i.e. the turnover of the erbB-2 protein following mAb binding. SKBR3 and HC11 clone R111 cells were metabolically labeled with [35S]methionine, then chased for 24 h with medium containing unlabeled methionine plus 10 μg/ml of each of the mAbs. The erbB-2 protein was immunoprecipitated, and the content of [35S]methionine-labeled p185 was analyzed by SDS-PAGE followed by autoradiography. The results of the experiment are shown in Fig. 6. The binding of mAbs FRP5, FSP16, and FSP77 accelerates the turnover of the erbB-2 protein in SKBR3 cells (panel A) and in clone R111 cells (panel B). In both cell lines the binding of mAb FWP51 has no effect upon the turnover of the erbB-2 protein.

FIG. 7. Tyrosine phosphorylation of cellular proteins in SKBR3 cells treated with erbB-2-specific antibodies and with EGF. SKBR3 cells were left untreated (lane 2) or treated 15 min at 37 °C with 10 μg/ml of mAb FRP5, FSP16, FWP51, and FSP77 (lanes 3–6) or with 100 ng/ml EGF (lane 1). Equal amounts of cell lysates were separated by SDS-PAGE, and a protein blotting analysis using the phosphotyrosine specific mAb was performed as described in Fig. 5. In lane 7, PLC-γ1 was immunoprecipitated from SKBR3 extracts with a specific mAb, and the precipitated material was run on the same gel. The PLC-γ1 was detected in a blotting analysis using the specific mAb. The positions of the p185 erbB-2 protein and the p145 PLC-γ1 protein are indicated on the right. The arrows on the left show the positions of the prestained protein markers of 180, 116, and 84 kDAs.
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3-6). protein was immunoprecipitated with the 21N antiserum. The control

cells following binding of the specific antibodies. SKBR3 cells

tions of mAb FSP77 were inhibited in their growth by 90%. The medium was changed every 2-3 days, and the cell number was determined at day 12 before the cultures reached confluence. The results are from duplicate wells, and the standard deviation is shown by the crosses.

We have examined the biological effects which the four erbB-2-specific antibodies have upon the receptor protein in intact living cells. Two different cell lines were analyzed: SKBR3, a human breast tumor cell line, and HC11 clone R111, a mouse mammary epithelial cell line transfected with a plasmid encoding the human erbB-2 protein. The results obtained with both cell lines were comparable with respect to the erbB-2 protein down-regulation. The turnover of the erbB-2 protein in intact cells is slightly stimulated following binding of mAbs FRP5, FSP16, and FSP77. In an experiment not shown, we determined that in untreated SKBR3 cells and in cells treated with the mAbs FRP5 and FSP16 the half-life of the erbB-2 protein was, respectively, 8 and 6 h. It is interesting that the binding of mAbs FRP5 and FSP16, which recognize similar domains on the erbB-2 protein, has the strongest effect upon the erbB-2 protein turnover.

With respect to the effects which binding of the mAbs had upon the phosphotyrosine content of the erbB-2 receptor, there were some differences observed between SKBR3 and R111 cells. Binding of all four of the antibodies to SKBR3 cells led to an increase in the phosphotyrosine content of the erbB-2 protein, although the effect of mAb FSP77 was weak. In R111 cells FSP77 did not effect the phosphotyrosine content of the receptor, while the other three mAbs had stimulatory effects. This difference could be due to the fact that the SKBR3 cells express approximately 10-fold more erbB-2 molecules than the R111 cells. The increased level of phosphotyrosine present in the erbB-2 receptor following mAb binding may reflect receptor activation via binding to the domain normally used by the ligand, or may reflect dimerization and ensuing activation via the bivalent binding of the antibody. This problem will be addressed using monovalent Fab fragments. A ligand for the erbB-2 protein has been described (41, 42) but is not yet readily available. Antibody
activation of the erbB-2 protein may help to circumvent this problem and prove useful, e.g., in studying short term effects of receptor activation or in assays to screen for agents which inhibit erbB-2 kinase activity.

The pattern of phosphoryrosine-containing proteins present in SKBR3 cells following treatment with the mAbs displayed differences when compared to unstimulated cells. This was most obvious in cells treated with FRP5 and FWP51, the two antibodies which stimulate the erbB-2 phosphorylation to the greatest extent. The elevated level of phosphoryrosine on intracellular proteins most likely reflects the activation of the receptor following antibody treatment of the cells. In fibroblasts it has been reported that the stimulated erbB-2 receptor is coupled to p145 PLC-γ1 phosphorylation and activation (45, 46). A protein which comigrates with PLC-γ1 displayed enhanced phosphoryrosine in cells treated with both mAbs, and we are currently examining the phosphoryrosine content of the isolated PLC-γ1. It is interesting that EGF treatment of the SKBR3 cells leads to a different pattern of phosphoryrosine-containing proteins, suggesting that these two receptors couple to different intracellular signaling pathways. This is in agreement with our previous publications describing these two receptors in mammary epithelial cells (14, 33) and from others who examined these receptors in fibroblasts and in transfected hematopoietic cells (46, 47).

The increased phosphoryrosine found in the erbB-2 and other cellular proteins following antibody binding, while suggesting receptor activation, did not result in a measurable increase in DNA synthesis in HC11 R111 cells. Similar results have been described for anti-EGF receptor antibodies. In one study it was found that the binding of three EGF receptor-specific mAbs to intact cells led to an increase in the phosphotyrosine content of the receptor, but none of the antibodies stimulated DNA synthesis (48). In contrast, there are two reports of anti-EGF receptor antibodies which behave as full ligand agonists (49, 50). One mAb competes with EGF for receptor binding (49) and the other does not (50), but both were able to stimulate kinase activity, receptor internalization, and DNA synthesis. None of the erbB-2-specific antibodies described in this paper appear to have full ligand agonistic activity.

The most striking result which we obtained was the growth inhibitory effect which mAb FSP77 had upon the SKBR3 cells. This antibody inhibits the anchorage-dependent growth of SKBR3 cells up to 90%. We have analyzed the effect of FSP77 upon the anchorage-dependent growth of SKBR3 cells. This antibody inhibits the anchorage-dependent growth of SKBR3 cells, does compete for binding with a protein which appears to be an erbB-2 ligand (43). Other erbB-2-specific mAbs have been reported to inhibit the proliferation of SKBR3 cells (40) or Calu-3 tumor cells which also express high levels of the receptor (39). It will be interesting to see if these antibodies bind to similar epitopes on the extracellular domain.

In summary, the four anti-erbB-2 antibodies behave as partial ligand agonists and one of them, FSP77, strongly inhibits the in vitro growth of SKBR3 breast tumor cells. Preliminary results show that FSP77 also inhibits nude mouse tumor growth of erbB-2-transformed cells. In a modified form these erbB-2-specific monoclonal antibodies may prove useful as therapeutic agents in the treatment of human malignancies.

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& Jackson, J. E. (1991) Cancer Res. 51, 2593-2598
39. Tagliabue, E., Centis, F., Campiglio, M., Mastroianni, A., Martignone, S., Pellegrini, R., Casalini, P., Lanzù, C., Menard, S. & Colnaghi, M. I. (1991) Int. J. Cancer 47, 933-937
40. Stancovski, I., Hurwitz, E., Leitner, O., Ullrich, A., Yarden, Y. & Sela, M. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 6921-6925
41. Drebin, J. A., Link, V. C. & Greene, M. I. (1988) Oncogene 2, 387-394
42. Yarden, Y. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 2569-2573
43. Lupu, R., Colomer, R., Zugenmaier, G., Sarup, J., Shepard, M., Slamova, D. & Lippman, M. E. (1990) Science 249, 1552-1555
44. Yarden, Y. & Peles, E. (1991) Biochemistry 30, 3543-3550
45. Peles, E., Levy, R. B., Or, E., Ullrich, A. & Yarden, Y. (1991) EMBO J. 10, 2077-2086
46. Fazioli, F., Kim, U.-H., Rhee, S. G., Molloy, C. J., Segatto, O. & Di Fiore, P. P. (1991) Mol. Cell. Biol. 11, 2040-2048
47. Di Fiore, P. P., Segatto, O., Tatlor, W. G., Aaronson, S. A. & Pierce, J. H. (1990) Science 248, 70-83
48. Defize, L. H. K., Moolenaar, W. H., van der Saag, P. T. & de Laat, S. W. (1991) EMBO J. 10, 2007-2014
49. Fazioli, F., Kim, U.-H., Rhee, S. G., Molloy, C. J., Segatto, O. & Di Fiore, P. P. (1991) Mol. Cell. Biol. 11, 2040-2048
50. Defize, L. H. K., Moolenaar, W. H., van der Saag, P. T. & de Laat, S. W. (1991) EMBO J. 10, 2007-2014
51. Peles, E., Levy, R. B., Or, E., Ullrich, A. & Yarden, Y. (1991) EMBO J. 10, 2077-2086
52. Ennis, B. W., Valverius, E. M., Bates, S. E., Lippman, M. E., Bellot, F., Kris, R., Schlessinger, J., Masui, H., Goldenberg, A., Mendelsohn, J. & Dickson, R. B. (1989) Mol. Endocrinol. 3, 1830-1838