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Regulation of Phosphorylation of the c-*erbB*-2/*HER2* Gene Product by a Monoclonal Antibody and Serum Growth Factor(s) in Human Mammary Carcinoma Cells

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Monoclonal antibody (MAb) 4D5 was used to analyze the phosphorylation of p185^{HER2}, the gene product of c-*erbB*-2/*HER2*, in SK-BR-3 cells. Culture in the continuous presence of 4D5 reduced the in vivo steady-state levels of p185^{HER2} phosphorylation by 80% in a dose-dependent manner, suggesting that MAb 4D5 may have interfered with the activation of phosphorylation of p185^{HER2}. The observed MAb-mediated reduction of p185^{HER2} phosphorylation could not be completely accounted for by down-regulation. When cultures were grown under serum-free conditions, the steady-state levels of p185^{HER2} phosphorylation were reduced by 56%, and addition of 4D5 further inhibited phosphorylation to 20% of steady-state levels. With continuous exposure to increasing concentrations of newborn calf serum in these cultures, there was a linear increase in tyrosine-specific phosphorylation of p185^{HER2}, reaching a 5.4-fold increase with 10% newborn calf serum. Phosphorylation of p185^{HER2} in the presence of newborn calf serum was not attributable to stimulation of the epidermal growth factor receptor by epidermal growth factor or by transforming growth factor-α. Extension of these observations to two other mammary carcinoma cell lines, MDA-MB-453 and BT-474, also demonstrated a significant capacity of serum to induce p185^{HER2} phosphorylation. The demonstration of antibody-mediated partial inhibition of phosphorylation under serum-free conditions suggests that mammary carcinoma cells may also produce and secrete a factor or factors which may activate p185^{HER2}. Our observation that growth-inhibitory MAb 4D5 is able to reduce the phosphorylation of p185^{HER2} by newborn calf serum and by a cellular-derived factor(s) suggests the existence of a growth factor(s) which uses phosphorylation of p185^{HER2} as a signal transduction pathway to regulate cell proliferation.

Proto-oncogenes are a group of normal genes which play important roles in the regulation of cell proliferation and function (2, 5). Abnormalities in the expression, structure, or activity of proto-oncogene products contribute to the development and maintenance of the malignant phenotype in complex but important ways (36, 37, 46). Evidence that the gene products of several activated proto-oncogenes are either growth factors or growth factor receptors has suggested a possible link between proto-oncogenes and growth factors (20). For example, the receptor for macrophage colony-stimulating factor is identical to the product of c-*fms* (35), and c-*erbB*-1 encodes the receptor for epidermal growth factor (13) and transforming growth factor-α (TGF-α) (43). Growth factor receptors encoded by proto-oncogenes are transmembrane glycoproteins with intrinsic tyrosine-kinase activity (22). Receptor tyrosine kinases are activated by binding of their respective ligands, the growth factors (48). This activity is thought to be an integral part of signal transduction processes involved in the regulation of cell proliferation (21). Overexpression of some growth factor receptors has been shown to induce transformed properties in recipient cells (11, 32), possibly because of excessive activation of signal transduction mechanisms. Furthermore, a number of tumor cells with increased expression of growth factor receptors also produce ligands for these receptors (10).

HER2 (also known as c-*erbB*-2 or c-*neu*), the human homolog of the rat proto-oncogene *neu* (9), encodes a 185-kDa transmembrane glycoprotein with intrinsic tyrosine kinase activity which is presumed to be the receptor for an as-yet-unidentified ligand (3, 39). p185^{HER2} also has homology to, but is distinct from, the epidermal growth factor receptor (EGF-R), which is the product of c-*erbB*-1. Both proteins have a cysteine-rich extracellular domain, a transmembrane domain, and an intracellular tyrosine kinase (4, 31, 47). In spite of sequence homology between c-*erbB*-2 and c-*erbB*-1, EGF does not bind to p185^{HER2} (33). p185^{HER2} has been shown to be overexpressed or amplified or both in a number of human malignancies: breast (45), ovarian (38), thyroid (1), lung (7), salivary gland (34), and stomach (50). In addition, p185^{HER2} is a potent oncogene capable of inducing transformation and tumorigenesis when overexpressed in NIH 3T3 cells (12, 19). Overexpression of p185^{HER2} also induces tumor cell resistance to macrophage killing (15). Thus p185^{HER2} may have an important role in the development and maintenance of human tumors.

These observations suggest that receptor-associated tyrosine kinase activity of overexpressed proto-oncogene protein products is important for the regulation of cell growth. We have developed a panel of monoclonal antibodies (MAbs) reactive with domains of the human EGF-R (23) and p185^{HER2} (17, 18) in intact cells and have demonstrated antiproliferative effects of these antibodies in vitro (16, 18, 23) and in vivo (29). Antibody 4D5, which is specifically directed against p185^{HER2}, exhibits strong antiproliferative activity on cultured human breast tumor cell lines which overexpress p185^{HER2} (18). Since p185^{HER2} is a receptor

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with intrinsic tyrosine kinase activity, we investigated the modulation of p185^{HER2} phosphorylation by MAb 4D5. We report here that activation of phosphorylation of p185^{HER2} by serum was reduced in the presence of an excess of MAb 4D5 and that MAb-sensitive phosphorylation was mediated by a growth factor or factors other than TGF-α or EGF. Furthermore, SK-BR-3 cell-conditioned medium contained a factor(s) that could activate p185^{HER2} phosphorylation and was partially inhibited by MAb 4D5.

MATERIALS AND METHODS

Materials. MAbs 4D5 (18) and 9G6 (44) were raised against human p185^{HER2}. MAbs 528 and 225 bind to the human EGF-R (23). Antiphosphotyrosine MAb PY-69 was obtained from ICN Biochemicals, Inc. Rabbit immunoglobulin to mouse immunoglobulins G (RAM) was supplied by Accurate Chemicals, Westbury, N.Y. ^{32}P$_i$ (carrier free; 28.5 Ci/nmol) and ^{35}S-labeled L-cysteine (1,030 Ci/mmol) were purchased from New England Nuclear, Boston, Mass.

Cell lines and cell culture. Human breast tumor cell lines SK-BR-3, BT-474, and MDA-MB-453 were obtained from the American Type Culture Collection. The A431 human epidermal carcinoma cell line was originally supplied by Gordon Sato. All cell lines except MDA-MB-453 (which was grown in L-15 medium) were maintained in Ham F-12–Dulbecco modified Eagle medium (1:1, vol/vol) (F-12/DMEM) supplemented with 10% fetal bovine serum.

Labeling of p185^{HER2} with ^{32}P$_i$ and [^{35}S]cysteine. Cells (3 × 10^5) were plated in F-12/DMEM in each well of a six-well dish. Twenty-four hours later, cultures were washed with phosphate-free medium and incubated for up to 15 h in phosphate-free F-12/DMEM containing 0.4 mCi of ^{32}P$_i$ per ml in the presence or absence of MAb and newborn calf serum. At desired times, cells were harvested in 400 μl of lysis buffer (20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.5], 1% Triton X-100, 10% glycerol, 1.5 mM magnesium chloride, 1 mM ethyleneglycol bis-N,N,N',N'-tetraacetic acid, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptine per ml, 2 mM sodium orthovanadate) at 4°C for 20 min. The lysate was centrifuged at 10,000 rpm in an Eppendorf microfuge for 10 min, and then 60 μl of Pansorbin was added as described elsewhere (42). For labeling with [^{35}S]cysteine, the cells were washed with cysteine-free medium and refed with cysteine-free F-12/DMEM containing 0.15 mCi of [^{35}S]cysteine per ml with or without 5% newborn calf serum.

Immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Aliquots (350 μl) of the cell lysates (or equal amounts of trichloroacetic acid-precipitable counts per minute) containing ^{32}P-labeled or [^{35}S]cysteine-labeled p185^{HER2} were subjected to immunoprecipitation with 10 μg of MAb 9G6, 528, or PY-69 at 4°C for 2 h. Immune complexes were collected by absorption to RAM-protein A-Sepharose beads at 4°C for 1 h. Beads were washed three times with 1 ml of buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 2 mM sodium orthovanadate). Washed pellets were mixed with 40 μl of sample loading buffer (10 mM Tris HCl [pH 6.8], 1% sodium dodecyl sulfate [SDS], 0.2% 2-β-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue), heated at 95°C for 5 min, and resolved on a 7% SDS-polyacrylamide slab gel (26). The efficiency of precipitating labeled receptor with MAb 9G6 is 80 to 90% when this procedure is used. Low-molecular-mass colored markers (Amersham Corp.) were used as standards.

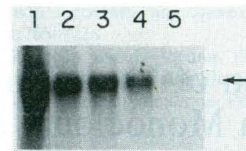**Phosphoamino acid analysis.** The band corresponding to

FIG. 1. Effect of MAb 4D5 on steady-state levels of p185^{HER2} phosphorylation in SK-BR-3 cells. Subconfluent cultures were labeled with ^{32}P$_i$ (400 μCi in 1 ml of phosphate free F-12/DMEM supplemented with 5% newborn calf serum) in the continuous presence of different amounts of antibody for 15 h. Detergent extracts were made, and p185^{HER2} was immunoprecipitated by using MAb 9G6 and then resolved by 7% SDS-polyacrylamide gel electrophoresis (Materials and Methods). An autoradiogram resulting from 16 h of exposure of the dried gel is shown here. The arrow indicates the position of ^{32}P-labeled p185^{HER2}. Lane 1, Control cells; lanes 2 to 4, cells treated with MAb 4D5 at 30, 150, and 300 nM, respectively; lane 5, cells treated with 400 nM F(ab) fragment of MAb 4D5. The amounts (in counts per minute) of p185^{HER2} in each lane were 4,453 (lane 1), 1,967 (lane 2), 1,785 (lane 3), 1,040 (lane 4), and 335 (lane 5). Counts were corrected by subtracting the background of 60 cpm. The results shown are representative of results in six different experiments.

the 185-kDa *HER2* protein, resolved as described above, was excised out of the gel. ^{32}P-labeled p185^{HER2} in a gel slice was partially hydrolyzed with 200 μl of 6 N HCl at 110°C for 1 h. Two portions (10 μl each) of the hydrolysate were taken for measurement of radioactivity in a liquid scintillation counter to determine the total incorporation of ^{32}P into the p185^{HER2} receptor. The rest of the hydrolysate was dried, suspended in distilled water, and applied to a Dowex AG1-X8 column. The column was washed with distilled water, and the absorbed ^{32}P-labeled materials were eluted with 0.5 N HCl and lyophilized. The recovery of radioactivity by this procedure was 78 to 85%. ^{32}P-phosphoamino acids mixed with unlabeled carrier phosphoamino acids (phosphoserine, phosphothreonine, and phosphotyrosine [1:1:1]) were analyzed by thin-layer electrophoresis as described elsewhere (8).

RESULTS

MAb 4D5 reduces amount of ^{32}P-labeled p185^{HER2}. MAb 4D5 was used to investigate the regulation of phosphorylation. SK-BR-3 cells, which have an amplified c-*erbB*-2 gene (45), were cultured for 15 h in medium containing ^{32}P$_i$ in the continuous presence of various concentrations of MAb 4D5. The p185^{HER2} from these cells was immunoprecipitated with another anti-p185^{HER2} MAb, 9G6, which recognizes a distinct epitope of p185^{HER2}, and resolved by SDS-polyacrylamide gel electrophoresis. Results of such an experiment are shown in Fig. 1. Treatment of cells with 4D5 reduced in vivo steady-state levels of ^{32}P-labeled p185^{HER2} up to 80% in a dose-dependent manner (lanes 2 through 4). There was 49% ± 8% reduction in phosphorylation by 150 nM MAb 4D5 in eight different experiments. When the F(ab) fragment of 4D5 was used instead of intact antibody, comparable or greater reduction of ^{32}P-labeled p185^{HER2} was observed (lane 5). As a control, SK-BR-3 cells were incubated with another MAb, 225 IgG1, specifically directed against the EGF-R, and there was no effect on the amount of ^{32}P-labeled p185^{HER2} (unpublished data). The reduction in steady-state levels of ^{32}P-labeled p185^{HER2} was not due to interference by 4D5 with MAb 9G6 during the immunoprecipitation reaction, as immunoprecipitation performed with another polyclonal an-
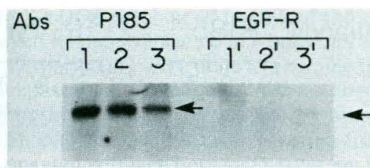
FIG. 2. Specificity of the reduction of ^{32}P-labeled p185^{HER2} by MAb in SK-BR-3 cells in the presence or absence of MAb 4D5. Subconfluent cells were labeled with ^{32}P$_i$ for 15 h. The cells were lysed in 600 μl of extraction buffer and divided into two equal parts of 250 μl each. Immunoprecipitation was performed with anti-p185 MAb (lanes 1 to 3) or with anti-EGF-R MAb 528 (lanes 1' to 3'). An autoradiogram of a dried gel is shown here. Lane 1 and 1', Control; lanes 2 and 2', 30 nM MAb 4D5; lanes 3 and 3', 150 nM MAb 4D5. Counts per minute: lane 1, 5,985; lane 2, 3,798; lane 3, 3,120; lane 1', 853; lane 2', 779; lane 3', 932. Abs, Antibodies.

tibody (18) recognizing the carboxy-terminal 17 amino acids of p185^{HER2} gave similar results (unpublished data).

Next, we examined the possibility of general inhibitory effects of MAb 4D5 on the steady-state levels of other ^{32}P-labeled receptor proteins by analyzing the amount of ^{32}P-labeled p185^{HER2} and ^{32}P-labeled EGF-R in the same experiment (Fig. 2). These results indicated that there was no reduction of ^{32}P-labeled EGF-R during 15 h of treatment of SK-BR-3 cells with 150 nM MAb 4D5, which had reduced the amount of ^{32}P-labeled p185^{HER} by 48%.

Analysis of reduction of p185^{HER2} phosphorylation. The reduction of steady-state levels of ^{32}P-labeled p185^{HER2} by MAb 4D5, shown in Fig. 1 and 2, could result from down-regulation of p185^{HER2} and/or interference in the activation of p185^{HER2} phosphorylation by a direct or indirect mechanism(s). In initial studies to explore these possibilities, parallel cultures of cells were metabolically labeled with [^{35}S]cysteine or ^{32}P$_i$. During 11 h of concurrent incubation with MAb 4D5, there was a 45% reduction in ^{32}P-labeled p185^{HER2} (Fig. 3A) and only a 14% reduction in ^{35}S-labeled p185^{HER2} (Fig. 3B). This suggests that the reduced ^{32}P label in p185^{HER2} in the presence of MAb 4D5 can only partially be attributed to reduced p185^{HER2} content. Next, we performed a similar experiment comparing the capacities of the monovalent F(ab) fragment of MAb 4D5 and an intact MAb 4D5 to affect the reduction of ^{35}S-labeled p185^{HER2}. There was no change in ^{35}S-labeled p185^{HER2} in the presence of F(ab), but there was a 26% reduction caused by MAb 4D5 (Fig. 3C, lanes 3 and 2, respectively). The results obtained in the immunoprecipitation experiments documented in Fig. 3A through C were confirmed by immunoblotting (D). Immunoblotting of the ^{32}P-labeled SK-BR-3 cell extracts used in Fig. 2 demonstrated only a marginal reduction in the content of p185^{HER2} protein when cells were cultured in the presence of MAb 4D5 but a substantial reduction in the amount of ^{32}P-labeled p185^{HER2} (Fig. 2). The expression of EGF-R was not affected. Immunoblotting of similar unlabeled SK-BR-3 extracts also demonstrated very little reduction in the content of p185^{HER2} by MAb 4D5 (Fig. 3D, experiment 2). These findings indicate that increased receptor catabolism induced by a MAb cannot fully account for the observed reduction in ^{32}P labeling and show [with F(ab)] that reduced labeling is dissociated from reduced content of p185^{HER2}.

Next we addressed the possibility that the reduction in ^{32}P-labeled p185^{HER2} associated with exposure to MAb 4D5 could be related to a change in the level of expression of p185^{HER2} on the plasma membrane or to the extent of
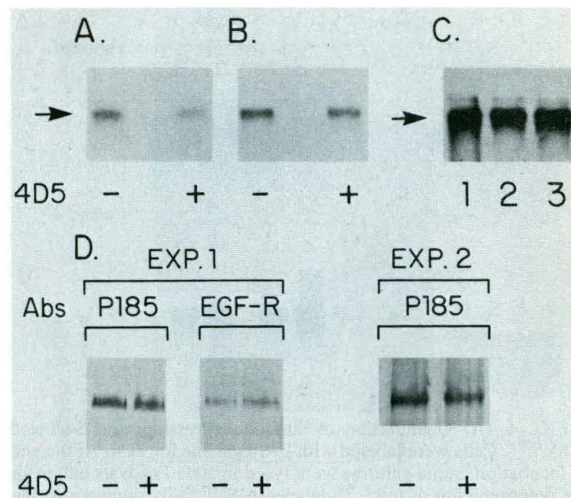

FIG. 3. Analysis of the reduction of p185^{HER2} phosphorylation in SK-BR-3 cells treated with MAb 4D5. Cells were labeled with ^{32}P$_i$ (A) or [^{35}S]cysteine (B) in the presence or absence of MAb 4D5 (150 nM) for 11 h. Samples were prepared and separated as described in Materials and Methods. The autoradiogram shown here was obtained by 6 h of exposure. (C) Cells were labeled with [^{35}S]cysteine for 11 h in the presence of MAb 4D5 (150 nM, lane 2) or F(ab) (400 nM, lane 3) or with culture medium (lane 1). Samples were prepared and immunoprecipitation was carried out as described in Materials and Methods. An autoradiogram of a dried gel is shown here. Quantitation of the p185^{HER2} bands was obtained by densitometric scanning (A through C) or by determining radioactivity associated with bands (A and B). Quantitation by determining the radioactivity associated with p185^{HER2} bands in panels A and B gave results similar to those with densitometric scanning, and there was a 27% ± 3% additional reduction in ^{32}P-labeled p185^{HER2} compared with ^{35}S-labeled p185^{HER2}. (D) Immunoblotting of p185^{HER2} and EGF-R proteins. In experiment 1 (Exp.1), ^{32}P-labeled SK-BR-3 cell extracts (50 μg of protein) used in Fig. 2, lanes 1 and 2, were resolved on a 7% SDS-polyacrylamide gel and then immunoblotted with anti-P185 MAb 9G6 or anti-EGF-R polyclonal antibody RK-II. Experiment 2 shows the immunoblotting of unlabeled SK-BR-3 cell extracts prepared following culture for 15 h with or without 30 nM MAb 4D5. Since some of the extracts used here were radiolabeled, immunoblotted membranes were visualized by using a protein A-gold enhancement kit (30). Abs, Antibodies.

down-regulation of receptor protein. First, we determined what fraction of the ^{35}S-labeled p185^{HER2} is present on the cell surface at 37°C (Fig. 4A). In these experiments, p185^{HER2} expressed on the plasma membrane was identified by its capacity to bind MAb 4D5 prior to cell lysis. The results indicate that 19% ± 4% (average from three different experiments) of total ^{35}S-labeled p185^{HER2} is expressed on the cell surface under these experimental conditions; thus p185^{HER2} is available for down-regulation by MAb 4D5. Down-regulation of EGF-R has been shown to be dependent on temperature (41). To confirm that down-regulation of surface p185^{HER2} also is reduced at 4°C, experiments were performed to analyze the effect of temperature on the abundance of ^{35}S-labeled p185^{HER2} on the cell surface. Results indicated that at 4°C the amount of total ^{35}S-labeled p185^{HER2} expressed on the surface increased to 35% ± 3% (data not shown) compared with 19% ± 4% of total ^{35}S-labeled p185^{HER2} at 37°C.

In order to define the contribution of down-regulation to MAb-induced reduction in p185^{HER2} phosphorylation, we

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