Journal of Cellular Biochemistry 67:316–326 (1997)

# Binding-Induced Activation of Overexpressed p185$^{HER2}$ Is Essential in Triggering Neuronal Differentiation of PC12 Cells

**Rosaria Orlandi,* Cristina Formantici, Maria I. Colnaghi, and Sylvie Ménard**

Division of Experimental Oncology E, Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy

**Abstract** To determine whether p185$^{HER2}$ overexpression per se triggers p185$^{HER2}$ cellular signaling or whether an extracellular signal is required, we transfected PC12 cells with the human erbB-2 proto-oncogene, and established a cell line that overexpresses p185$^{HER2}$. PC12-HER2 cells, maintained in suspension culture or plated on a collagen layer, showed the same morphology and growth rate as PC12 and PC12 mock-transfected control cells. When treated with monoclonal antibody (MAb) MGr6 or other anti-p185$^{HER2}$ MAbs, PC12–HER2 cells specifically underwent neuronal differentiation comparable to that induced by nerve growth factor (NGF), and the differentiation-inducing effect of the MAb was dramatically enhanced by the addition of a second anti-mouse IgG. MAb-induced cell differentiation correlated with p185$^{HER2}$ phosphorylation, recruitment of Shc and Grb-2 transducer molecules into complexes, and MAPK phosphorylation. These data indicate the requirement for a specific binding-induced activation of the overexpressed p185$^{HER2}$ receptor in inducing PC12 cell differentiation. PC12-HER2 cells represent a suitable system for selection of p185$^{HER2}$-activating ligands (peptides, phage-displayed peptides or proteins) or specific inhibitors of its tyrosine kinase activity. J. Cell. Biochem. 67:316–326, 1997. 

**Key words:** erbB-2/neu; p185$^{HER2}$ overexpression; PC12; cell differentiation; monoclonal antibodies

p185$^{HER2}$, encoded by the human erbB-2 proto-oncogene, is a member of a family of transmembrane receptor tyrosine kinases that include erbB-3, erbB-4 and the erbB-1 or epidermal growth factor (EGF) receptor [see Hynes et al., 1994; Dougall et al., 1994, for reviews]. The erbB-2 gene was initially identified as an activated oncogene in rat neuroblastomas induced by chemical mutagenesis of rat embryos [Padhy et al., 1982; Shih et al., 1981]. In those tumors, erbB-2 differs from the proto-oncogenic form by a single point mutation within the transmembrane domain of the receptor protein [Bargmann et al., 1996]. The human homologue of the rat erbB-2 gene was subsequently identified and mapped to chromosome 17 (q12-q22) [King et al., 1985; Coussens et al., 1985]. In normal human cells, erbB-2 is present as a single copy [Kallioniemi et al., 1992] and the human p185$^{HER2}$ protein is weakly detectable by immunohistochemical staining in epithelial cells of many normal tissues [Press et al., 1990]. Amplification of the proto-oncogene and overexpression of the apparently normal p185$^{HER2}$ have been detected with high frequency in human adenocarcinomas of several origins, and the association of p185$^{HER2}$ overexpression with poor prognosis has been reported in breast, ovarian, and lung carcinoma [Hynes et al., 1994; Kern et al., 1990; Slamon et al., 1989]. Owing to the differential expression of p185$^{HER2}$ on normal cells versus tumors, and the homogeneous expression of the antigen in positive tumors, the p185$^{HER2}$ receptor has been considered a tool for immunodiagnosis and a potential target for immunotherapy in ovarian and breast cancer [Hynes et al., 1994; Dougall et al., 1994]. Murine monoclonal antibodies (MAbs) directed against different epitopes on the extracellular domain of p185$^{HER2}$ have been generated, which are capable of either stimulating or inhibiting proliferation in vitro [Xu et al., 1993; Harwerth et al., 1992; Bacus et al., 1992; Tagliabue et al., 1991; Drebin et al., 1986] and preventing tumor development in vivo [Katsumata et al., 1995]. The mechanisms by which p185$^{HER2}$ is acti-

Contract grant sponsors: PF ACRO-CNR and AIRC/FIRC.

*Correspondence to: R. Orlandi, Division of Experimental Oncology E, Istituto Nazionale per lo Studio e la Cura dei Tumori, Via Venezian 1, 20133 Milan, Italy.
E-mail: Orlandi@istitutotumori.mi.it

Received 23 June 1997; Accepted 24 June 1997

vated, including binding to ligands, transregulation by other receptors, mutation, and overexpression, have been widely investigated [reviewed in Hynes et al., 1994].

A family of molecules called heregulins, which are expressed by the same gene and related to EGF, i.e., heregulin, Neu differentiation factor (NDF), acetylcholine receptor-inducing activity (ARIA), and glial growth factors, has been shown to induce p185HER2 tyrosine kinase activity [Marchionni et al., 1993; Falls et al., 1993; Lupu et al., 1990; Peles et al., 1992; Holmes et al., 1992], although direct binding of these factors to p185HER2 molecules was not demonstrated. Heregulins bind to the closely related p160HER3 and p180HER4 [Plowman et al., 1993; Tzahar et al., 1994; Carraway III et al., 1994], but not to the p185HER2 receptor itself [Sliwkowski et al., 1994; Plowman et al., 1993; Peles et al., 1993]. Activation of p185HER2 by these various ligands through different receptors raises the possibility that p185HER2 biological signalling involves oligomeric hetero-receptor assemblies [Carraway III et al., 1994a; Sliwkowski et al., 1994a; Peles et al., 1993a]. In addition to the heregulin family of ligands, other factors have been described that activate p185HER2 [Huang et al., 1992; Dobashi et al., 1991].

Data have accumulated on the ability of p185HER2 to transform cells in vivo and in vitro when overexpressed, and a direct involvement of overexpression in transformation has been suggested [Pierce et al., 1991; Hudziak et al., 1987; Di Fiore et al., 1987]. The fact that no oncogenic mutation of p185HER2 has been found associated with human neoplasia supports this hypothesis. However, it has recently been reported that p185HER2 is activated only in a subset of tumors overexpressing the receptor [DiGiovanna et al., 1995].

Activation of p185HER2 kinase triggers the Ras/MAPK signaling pathway via Shc/Grb-2 molecules [Marte et al., 1995; Ricci et al., 1995; Segatto et al., 1993; Janes et al., 1994] and either mitogenic or differentiating signals are transduced depending on the cellular context. To date, it is unclear whether the physiological function of p185HER2 is to promote proliferation or differentiation in vivo.

In the present study, we analyzed the effect of p185HER2 overexpression on cell differentiation in the absence or presence of an activating binding molecule. We find that p185HER2 overexpression per se is ineffective in inducing differentiation of PC12 cells [Greene et al., 1976] transfected with the human erbB-2 proto-oncogene and that only the specific activation of p185HER2 by MAbs leads to neurite outgrowth of the transfected cells. This system might be useful in the selection of p185HER2 activating ligands or specific inhibitors of its tyrosine kinase activity.

## MATERIALS AND METHODS

### Monoclonal Antibodies

Murine MAbs MGr6 (IGg2), initially designated MGr3, and MGr2 (IGg1) were raised against the p185HER2 extracellular domain [Centis et al., 1992; Tagliabue et al., 1991] and purified from ascites fluid of hybridoma-bearing nu/nu mice by affinity chromatography on protein A–Sepharose (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.

### Cell Lines and Transfection

Rat pheochromocytoma cell line PC12 was obtained from the American Type Culture Collection (ATCC; Rockville, MD) and maintained in RPMI 1640 medium (Microbiological Associates, Walkersville, MD) supplemented with 10% horse serum (GIBCO BRL, Gaithersburg, MD), 5% fetal calf serum (FCS), penicillin, and streptomycin (100 IU/ml).

PC12 cells were transfected by electroporation using the plasmid LTR-2/erbB-2 [Di Fiore et al., 1987], which contains the human erbB-2 cDNA under the control of the Moloney murine leukemia virus long terminal repeat. Electroporation was performed as described [Greco et al., 1993]. PC12-conditioned medium containing mycophenolic acid (5 μg/ml), xanthine (250 μg/ml) and hypoxanthine (20 μg/ml) was used for selection. Twenty days after transfection, several clones were recovered and screened by immunofluorescence using MGr2 and MGr6. Strongly positive cells were selected using magnetic cell sorting (Miltenyi Biotec GmbH, Sunnyvale, CA). Briefly, cells were incubated with MGr2-coated microbeads and subsequently separated according to the manufacturer's instructions. The percentage of p185HER2-positive cells and the expression level of eluted cells were analyzed by immunofluorescence assay.

A control plasmid was prepared from the LTR-2/erbB-2 vector, deleting the erbB-2 gene

by *XhoI* restriction. Transfection of PC12 cells and selection were carried out as described above.

PC12 cells transfected with the human erbB-2 cDNA (PC12-HER2) and PC12 mock-transfected cells (PC12-CF) were maintained in RPMI 1640 medium supplemented with 5% FCS, 10% horse serum (GIBCO), penicillin and streptomycin (100 IU/ml), mycophenolic acid (5 µg/ml), xanthine (250 µg/ml), and hypoxanthine (20 µg/ml).

SK-BR-3 and SK-OV-3 human carcinoma cell lines were obtained from ATCC and were maintained in RPMI 1640 medium supplemented with 10% FCS and antibiotics as above.

### Cell Differentiation Assay

To analyze the effect of MAbs and NGF on cell differentiation, PC12 parental or PC12-transfected cells were plated in 96- or 48-well plastic plates coated with 10 µg/ml collagen followed by addition of MAbs (2.5–20 µg/ml) or mouse NGF (50 ng/ml, 2.5 S form, Boehringer Mannheim, Mannheim, Germany) 24 h later; cell morphology was evaluated after 3 days by light microscopy. In experiments using MAb and second antibody, MAb was removed after 3 h of incubation, and affinity-purified goat antimouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added. Cells adherent on the collagen layer were treated essentially as described [Greco et al., 1993] with MAb or NGF in the presence of 200 nM K252a (Calbiochem, San Diego, CA), an alkaloid that specifically inhibits the TRK tyrosine kinase [Berg et al., 1992].

### Immunofluorescence Assay

Suspended cells were incubated for 1 h at 37°C with MAb 10 µg/ml. After washing, cells were further incubated with fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (Meloy, Springfield, VA) for 1 h at 0°C and analyzed by microscopy or with a FACScan (Becton Dickinson Immunocytometry System, San Jose, CA).

### Immunoperoxidase Assay

Differentiated cells adherent on slide chambers (GIBCO) were fixed with methanol and incubated with aVX antiserum [Possenti et al., 1989], directed against the VGF8a gene product (a kind gift of R. Possenti), which is induced by NGF treatment. The Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used for detection according to the manufacturer's instructions.

### Immunoprecipitation and Western Blotting

PC12-HER2 cells in suspension were placed in low-serum RPMI (1% FCS) for 24 h and subsequently incubated with MAb MGr6 10 µg/ml for 30 min at 37°C, washed and incubated further with antimouse IgG for 15 min at 37°C. Plated cells were incubated with MGr6 10 µg/ml for 3 h at 37°C followed by goat antimouse IgG for 3 days. Cells ($5–10 \times 10^6$) were lysed in 500 µl of RIPA buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP40, 1 mM $NaVO_4$, 2 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml pepstatin) for 20 min at 0°C. Soluble proteins were separated by centrifugation for 15 min at 13,000 rpm, and protein concentration was determined by Coomassie Plus Protein Assay (Pierce, Rockford, IL). Immunoprecipitations were carried out by incubating 3 mg of cell lysate with anti-Shc antibody (polyclonal rabbit; UBI, Lake Placid, NY) adsorbed on protein A–Sepharose (Pharmacia) or MGr2 absorbed on protein G–Sepharose for 2 h at 4°C. Immune complexes were washed 3 times with RIPA buffer, eluted, and denatured by heating for 5 min at 95°C in reducing Laemmli buffer. Immunoprecipitates or 60 µg of total cell lysate were resolved on SDS–polyacrylamide gels and transferred to nitrocellulose filters. After overnight blocking with 5% BSA in TBS (50 mM Tris-HCl pH 7.4, 150 mM NaCl) at 4°C, filters were probed for 2 h at room temperature with anti-c-neu (Oncogene Science, Manhasset, NY), anti-phosphotyrosine (UBI), anti-Shc (UBI), or anti-Grb (UBI) antibodies. After extensive washing, immune complexes were detected with $^{125}$I-anti-mouse Ig or $^{125}$I-protein A (Amersham, Little Chalfont, UK).

MAPK phosphorylation was evaluated using the PhosphoPlus MAPK Antibody Kit (New England Biolabs, Beverly, MA). Suspended PC12-HER2 cells were maintained in RPMI without FCS for 24 h and subsequently incubated with NGF for 5 min or with 10 µg/ml MGr6 for 5 min, washed, and incubated further with antimouse IgG for 5 min at 37°C. Cells were lysed with Laemmli buffer, boiled for 5 min and proteins were resolved by electrophoresis on an 11% SDS-polyacrylamide gel, and blotted. Nitrocellulose filters were reacted with antiphospho-

specific MAPK and anti p44/42$^{MAPK}$ antibodies followed by AP-conjugated second antibody and chemiluminescence.

## RESULTS

### Expression of the Human erbB-2 Gene in PC12 Cells

PC12 cells transfected with the LTR-2/erbB-2 plasmid containing the human erbB-2 cDNA [Di Fiore et al., 1987], or the control plasmid gave rise to mycophenolic-resistent clones of tightly packed, undifferentiated cells. No single differentiated cells were detected. Of 22 clones screened by immunofluorescence with MGr2 or MGr6, 8 expressed the human p185$^{HER2}$. Neither MAb recognized PC12 parental cells, whereas a MAb directed against the product of the rat erbB-2 gene showed slight reactivity with PC12 cells (not shown), suggesting low-level expression of this receptor on the cell surface, consistent with previous observations [Oshima et al., 1995].

A positive clone processed by magnetic cell sorting gave rise to a homogeneous cell population of 90–100% strongly MGr2-positive cells, designated PC12-HER2. These cells, maintained in suspension, showed the same morphology and growth rate as untransfected and undifferentiated PC12 cells or PC12-CF mock-transfected cells obtained by mycophenolic selection of PC12 cells transfected with the control plasmid.

FACS analysis of p185$^{HER2}$ expression levels based on reactivity with MGr2 indicated that mock-transfected and parental PC12 cells were negative, whereas PC12-HER2 cells were highly positive for MGr2 reactivity (Fig. 1). MGr2 reactivity of PC12-HER2 cells was higher than that of MCF7 cells, which express low levels of p185$^{HER2}$ on the cell surface (mean fluorescence, 530 versus 38), but comparable to that of SK-BR-3 and SK-OV-3 cells, which overexpress p185$^{HER2}$ (mean fluorescence, 218 and 446, respectively).

### Antibody-Induced Differentiation of PC12-HER2 Cells

PC12-HER2 cells and control PC12-CF mock-transfected cells plated on collagen maintained a morphology and growth rate indistinguishable from those of parental PC12 cells. No significant spontaneous neurite outgrowth was observed.

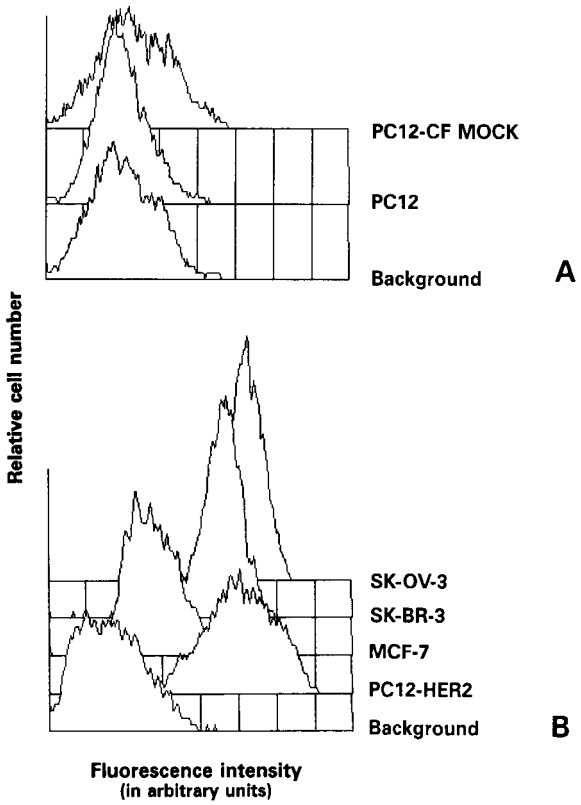


**Fig. 1.** p185$^{HER2}$ expression levels in PC12–HER2 cells. Flow cytometry analysis by indirect immunofluorescence of PC12-CF mock-transfected and parental PC12 cells **(A)**, and PC12-HER2, MCF-7, SK-BR-3, and SK-OV-3 cells **(B).** MCF7 cells express low levels of p185$^{HER2}$, whereas SK-BR-3 and SK-OV-3 cells overexpress the receptor. Cells were incubated with MGr2 and FITC-conjugated goat antimouse IgG or FITC-conjugated goat antimouse IgG alone to determine background staining.

NIH/3T3 cells transfected with the human erbB-2 cDNA [Di Fiore et al., 1987], which showed p185$^{HER2}$ expression levels comparable to that of PC12–HER2 cells (mean fluorescence of 572 by FACS analysis with MGr2), displayed a transformed phenotype (not shown).

To determine whether dimerization of the p185$^{HER2}$ induced by a bivalent antibody can activate the receptor and promote differentiation, PC12-HER2 cells were plated on collagen and treated with MGr6 alone or with MGr6 plus anti-mouse IgG. MGr6 specifically recognizes p185$^{HER2}$ and does not react with other molecules of the EGF receptor family, including erbB-1, erbB-3, and erbB-4 gene products ([Tagliabue et al., 1991] and data not shown). MGr6 treatment produced few differentiated cells and short neurites (Fig. 2a). By contrast, the addition of second antimouse antibodies remarkably increased the number of differentiated cells

and the length of neurites; neurites 5–10 times longer than the diameter of cell bodies were often observed (Figs. 2b, 3a,b). MGr6-induced differentiation was detectable at MAb concentrations of 2.5 µg/ml and was maximum at 5–10 µg/ml. No differentiation was observed when cells were maintained in the absence of antibodies or in the presence of the second antibody alone (Fig. 2c).

The morphology and kinetics of differentiation observed in MGr6 plus antimouse IgG-treated PC12-HER2 cells were very similar to those induced by NGF treatment (Fig. 2d). However the number of differentiated cells after MAb treatment was slightly lower than that produced by NGF treatment and several undifferentiated round-shaped cells persisted after MGr6 treatment.

PC12-CF mock-tranfected cells treated with MGr6 (Fig 2e) or MGr6 plus second antibodies (Fig. 2f) remained undifferentiated, like the untreated control (Fig. 2g), whereas neurite-bearing cells were observed after NGF treatment (Fig. 2h). PC12-HER2 cells incubated with MAb directed against the laminin receptor, an unrelated molecule expressed on the PC12 cell surface [Martignone et al., 1992], also gave rise to undifferentiated cells.

Several other anti-$p185^{HER2}$ MAbs, commercially available or produced in our laboratory, including MGr2, induced differentiation of PC12–HER2 cells but not of parental PC12 or PC12-CF mock-transfected cells (not shown). In addition, clones T9, D5, and D7 from PC12 cells transfected with the LTR-2/erbB-2 plasmid, which expressed levels of $p185^{HER2}$ comparable to that of PC12-HER2 cells, also differentiated after MGr6 plus anti-mouse IgG treatment, essentially as shown in Figure 2.

Immunoperoxidase staining of PC12-HER2 cells induced to differentiate by MGr6 treatment was carried out with an antiserum (aVX) directed against the product of the VGF8a gene, which is induced by NGF treatment. PC12-HER2 cells were positive for the VGF8a product after treatment with MGr6 alone or in the presence of second anti-mouse IgG at levels comparable to those observed in PC12 cells after NGF treatment (not shown).

K252a, a specific inhibitor of TRK tyrosine kinase, did not affect the neurite outgrowth of PC12-HER2 cells treated with MGr6 and second antibodies (Fig. 3a,b), but strongly inhibited the differentiation of PC12-HER2 cells treated with NGF (Fig. 3c,d). These results exclude the involvement of proto-TRK and NGF in the differentiation of antibody-treated PC12–HER2 cells.

### Antibody-Induced Signaling of $p185^{HER2}$ in PC12–HER2 Cells

To verify that the MAb-induced differentiation of PC12-HER2 cells was due to activation of $p185^{HER2}$ and triggering of a cellular signaling, tyrosine phosphorylation of $p185^{HER2}$ and its association with Shc transducer molecules, Grb-2 involvement and MAPK phosphorylation were analyzed. Lysates were prepared from PC12–HER2 cells treated with MGr6 and second antibodies or phosphate-buffered saline (PBS), separated on SDS–PAGE and immunoblotted either with antibodies to phoshotyrosine to determine the protein phosphotyrosine content, or with anti-$p185^{HER2}$ antibody to determine the level of protein expression. $p185^{HER2}$ proteins from MGr6-treated PC12–HER2 cells contained higher levels of phosphotyrosine than those from control cell lysates, despite comparable levels of $p185^{HER2}$ expression. The difference in phosphotyrosine content was also detected in differentiated cells 3 days after MGr6 treatment (Fig. 4). Lysates from PC12-CF mock-transfected cells showed no bands when immunoreacted with anti-$p185^{HER2}$ antibody (not shown). The exclusive specificity of MGr6 for $p185^{HER2}$, and not related receptors, ensured that the phosphorylated proteins detected were $p185^{HER2}$ and not homologous molecules of similar size.

Lysates from PC12-HER2 cells treated with MGr6 plus second antibodies or PBS were immunoprecipitated with MGr2, immunoblotted and reacted with anti-$p185^{HER2}$ and anti-Shc antibodies. $p185^{HER2}$ and $p52^{Shc}$ molecules were co-precipitated from MGr6-treated samples, but only $p185^{HER2}$ was precipitated from untreated control samples (Fig. 5A), indicating that MAb treatment induces a physical association between $p52^{Shc}$ and the intracellular domain of $p185^{HER2}$, and consistent with the MAb-induced increase in $p185^{HER2}$ phosphorylation. Similarly, MGr6- or PBS-treated lysates from PC12-HER2 cells immunoprecipitated with anti-Shc antibodies and analyzed by Western blot with anti-Shc and anti-Grb-2 antibodies revealed the co-precipiation of Shc and Grb-2 molecules in MGr6-treated, but not in control samples (Fig. 5B), suggesting that the formation of Shc-Grb-2

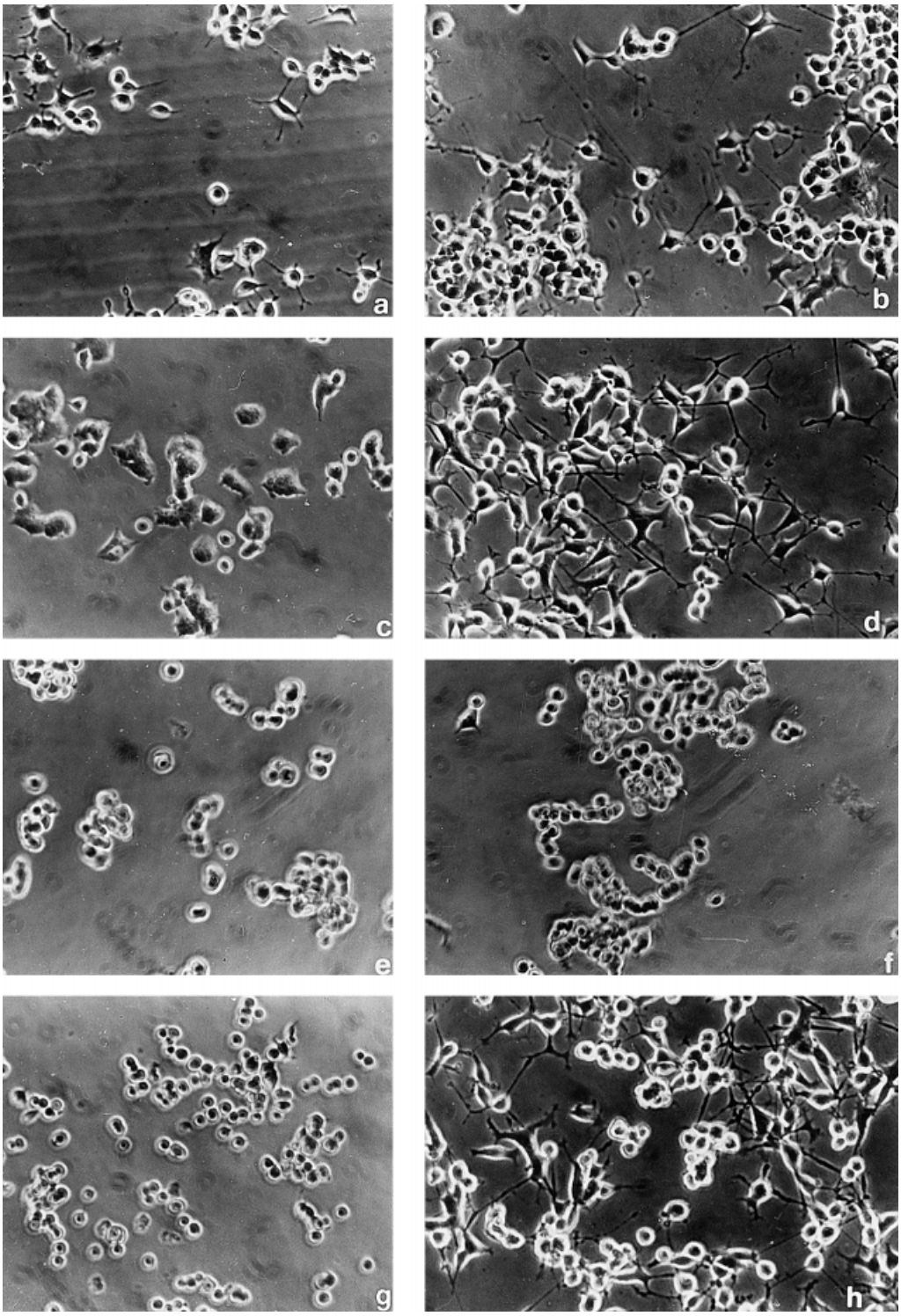


**Fig. 2.** Effect of MAb MGr6 on transfected PC12 cells. PC12–HER2 **(a–d)** or PC12-CF mock-transfected **(e–h)** cells were plated on collagen, treated with 10 μg/ml MGr6 **(a,e)**, 10 μg/ml MGr6 plus goat antimouse IgG **(b,f)** goat antimouse IgG alone **(c,g)**, or NGF **(d,h)** and photographed after 3 days.

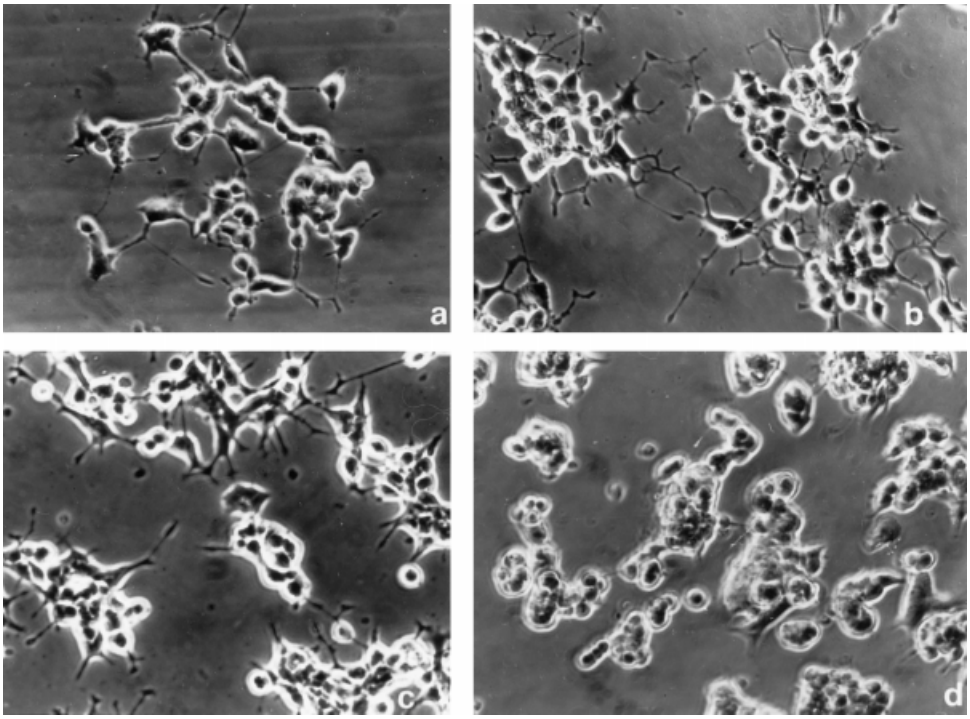


**Fig. 3.** Effect of K252a treatment on differentiation of PC12–HER2 cells. Cells were treated with 10 μg/ml MGr6 plus goat anti-mouse IgG **(a,b)** or NGF **(c,d)** in the absence **(a,c)** or presence of 200 nM K252a **(b,d)** and photographed after 3 days.

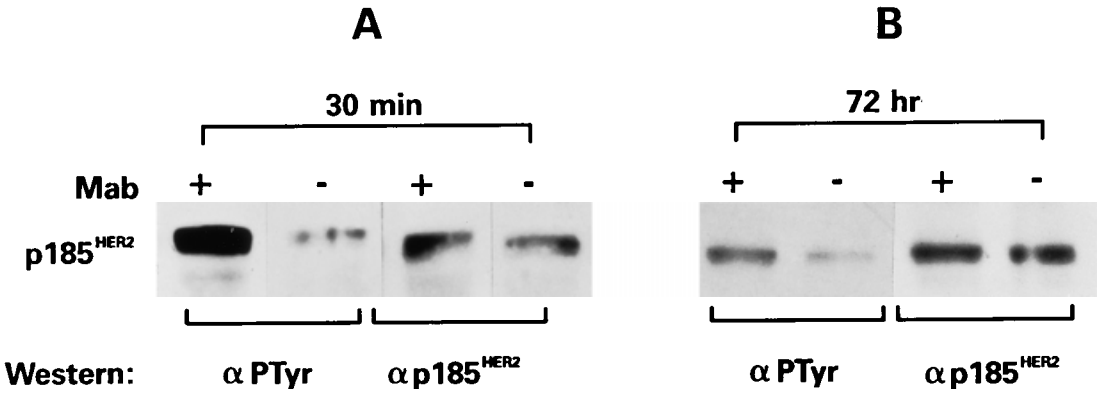


**Fig. 4.** Antibody-induced phosphorylation of $p185^{HER2}$. Undifferentiated cells were serum-starved and treated in suspension with 10 μg/ml MGr6 plus second antibody (+) or PBS (−) for 30 min and lysed **(A)**, or plated on collagen, treated with 10 μg/ml MGr6 plus second antibody (+) or PBS (−) and lysed after 72 h **(B)**. $p185^{HER2}$ proteins were resolved on a 7.5% SDS–polyacrylamide gel and immunoblotted with anti-phosphotyrosine antibodies and with anti-$p185^{HER2}$ antibody.

complexes is also associated with MAb-induced activation of $p185^{HER2}$.

Phospho-specific MAPK antibodies specifically recognized the phosphorylated tyrosine at position 204 of $p44^{MAPK}$ and $p42^{MAPK}$ in lysates from PC12–HER2 cells treated with MGr6 plus second antibodies or PBS (Fig. 6A). MAPK proteins from MGR6-treated PC12–HER2 cells were phosphorylated at levels comparable to those isolated from NGF-treated PC12–HER2 cells. No phosphorylation was detected in samples from PBS-treated PC12-HER2 cells. MAPK proteins from PC12-CF mock-transfected cells were not phosphorylated when cells were treated with MGr6 plus second antibodies or PBS, whereas phosphorylation was clearly

detected when cells were treated with NGF. All analyzed samples showed comparable levels of MAPK protein expression (Fig. 6B).

## DISCUSSION

Overexpression of normal p185$^{HER2}$ in human tumors has been widely reported and associated with tumor development. In vitro, p185$^{HER2}$ overexpression appears to be sufficient to transform NIH/3T3 cells [Hudziak et al., 1987; Di Fiore et al., 1987]. Signaling activation of overexpressed p185$^{HER2}$ was thought to result from the higher frequency of inter-receptor collisions, leading to a critical increase in the amount of p185$^{HER2}$ in oligomeric form [Hynes et al., 1994; Dougall et al., 1994]. Nevertheless, NDF/heregulin transcripts were found in NIH/3T3 cells [Alimandi et al., 1995], raising the possibility of an autocrine loop involving this ligand and p160$^{HER3}$ in signaling of overexpressed p185$^{HER2}$. Overexpression of oncogenic and proto-oncogenic forms of the rat erbB-2 gene product in transgenic mice [Dougall et al., 1994], together with the detection of activated p185$^{HER2}$ in only a subset of breast tumors overexpressing the receptor [DiGiovanna et al., 1995], suggest that other factors in vivo such as a ligand able to directly or indirectly activate

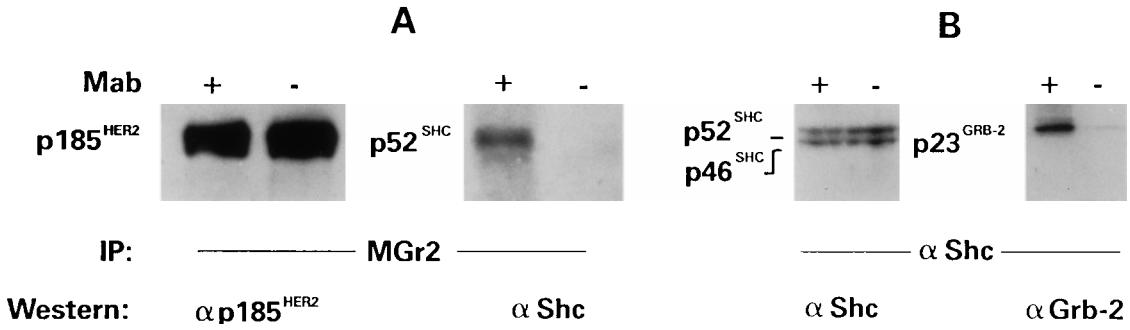


**Fig. 5.** Antibody-induced formation of Shc-p185$^{HER2}$ and Shc-Grb-2 complexes. Serum-starved PC12–HER2 cells treated in suspension with 10 µg/ml MGr6 and second antibody (+) or PBS (−) for 30 min were lysed. Lysates were immunoprecipitated with MGr2, separated on a 7.5% SDS–polyacrylamide gel, blotted and filters were reacted with anti-p185$^{HER2}$ and anti-Shc antibodies to detect p185$^{HER2}$-associated p52$^{Shc}$ molecules **(A)**, or immunoprecipitated with anti-Shc antibodies, separated on a 10% SDS-polyacrylamide gel, blotted and filters were reacted with anti-Shc and anti-Grb-2 antibodies to detect Shc-associated p23$^{Grb-2}$ molecules **(B)**.

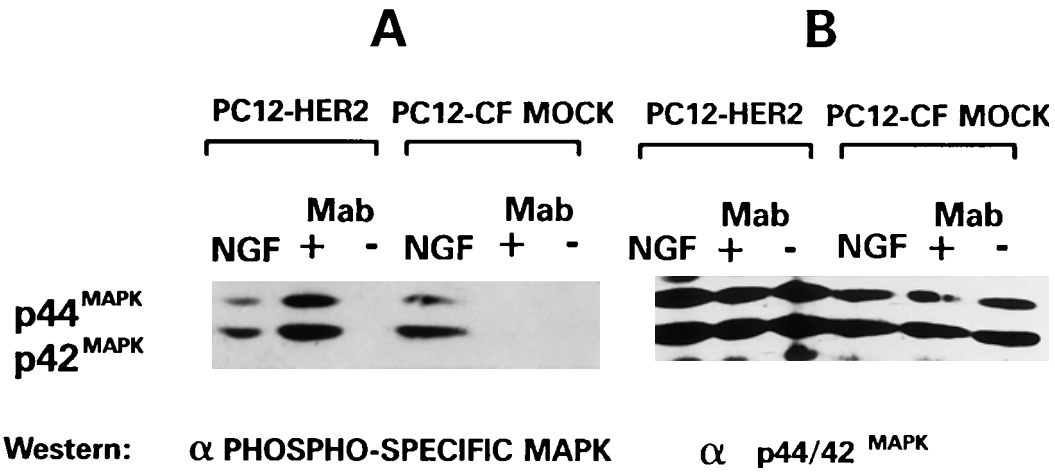


**Fig. 6.** Antibody-induced phosphorylation of MAPK. Serum-starved PC12-HER2 or PC12-CF mock-transfected cells treated in suspension with NGF, 10 µg/ml MGr6 and second antibody (+) or PBS (−) for 5 min were lysed, separated on an 11% SDS–polyacrylamide gel and blotted. Filters were reacted with anti-phospho-specific MAPK Ig **(A)**, which recognizes the phosphorylated form of p44$^{MAPK}$ and p42$^{MAPK}$ proteins, and with anti-p44/42$^{MAPK}$ Ig **(B)**.

$p185^{HER2}$ may be involved in triggering the abnormal signaling pathway of $p185^{HER2}$ in tumor cells.

To investigate whether $p185^{HER2}$ overexpression per se is effective in triggering $p185^{HER2}$ cellular signaling or whether the binding of an extracellular molecule is required, we analyzed the potential of overexpressed $p185^{HER2}$ to induce differentiation of PC12 cells in the presence or abscence of a specific binding activator.

Of several oncogenes previously transfected into PC12 cells, expression of those involved in the NGF-protoTRK signal transduction pathway has been shown to induce cell differentiation [Wood et al., 1993]. The erbB-2 proto-oncogene product also transduces mitogenic or differentiating signals through the same ras/MAPK pathway, and, when expressed in PC12 cells, induces differentiation [Gamett et al., 1994]. In our study, PC12 cells transfected with the LTR2/erbB-2 construct [Di Fiore et al., 1987], which contains the human proto-oncogene erbB-2 and is known to induce overexpression of $p185^{HER2}$ in NIH-3T3 cells followed by cell transformation, overexpressed $p185^{HER2}$ molecules at levels comparable to those of SK-BR-3 and SK-OV-3 cell lines. PC12-HER2 cells maintained in suspension or plated on collagen showed a morphology and growth rate comparable to those of PC12 and PC12 mock-transfected control cells. The $p185^{HER2}$ signaling pathway in PC12-HER2 cells was not activated, since no complexes involving Shc and Grb-2 molecules were detected and MAPK proteins were not phosphorylated. These findings suggest that overexpressed $p185^{HER2}$ in PC12 cells does not transduce any differentiating signal.

In the absence of a known ligand able to directly activate $p185^{HER2}$, we induced $p185^{HER2}$ dimerization using the stimulating MAb MGr6, which specifically recognizes $p185^{HER2}$, but not homologous molecules. When treated with MGr6, PC12–HER2 cells underwent neuronal differentiation, and this effect was dramatically enhanced by the addition of a second antibody, consistent with our previous observations [Srinivas et al., 1993]. Presumably, MGr6 binding to cell surface receptors is mainly monovalent; the addition of a second antibody induced clustering of MGr6-receptor complexes and, in turn, activation of $p185^{HER2}$. MGr6-induced differentiation was specific, since it was not observed after treatment of PC12–HER2 cells with MAb directed against an unrelated cell surface protein or in PC12-CF mock-transfected cells treated with anti-$p185^{HER2}$ MAb. In addition, several other anti-$p185^{HER2}$ MAbs, including MGr2, specifically induced differentiation of PC12-HER2 cells and other clones from PC12 cells transfected with the LTR-2/erbB-2 plasmid, which expressed levels of $p185^{HER2}$ comparable to that of PC12–HER2 cells, also differentiated after MGr6 plus anti-mouse IgG treatment.

MGr6-induced differentiation of PC12–HER2 cells was indistinguishable from that induced by NGF with respect to cell morphology. Moreover, the VGF8a gene product was expressed in MGr6-induced differentiation, as it is during NGF induction. However, NGF and the TRK receptor are not involved in MGr6-induced differentiation, since PC12–HER2 cell differentiation sustained by MGr6 was unaffected by K252a, whereas NGF-induced differentiation was markedly inhibited by this alkaloid.

The number of differentiated PC12–HER2 cells after MAb treatment was slightly lower than that after NGF treatment, and several undifferentiated, rounded cells persisted during MGr6 treatment. It remains unclear whether this reflects the low differentiation-inducing efficiency of MGr6 or a persistent proliferative activity in $p185^{HER2}$-transfected cells. In fact, PC12 cells that differentiate or proliferate have been observed after transfection of constitutively activated oncogenes such as TRK-T1 (Orlandi and Greco, unpublished results) or the mutated form of the rat erbB-2 oncogene [Gamett et al., 1994].

The MAb-induced differentiation of PC12–HER2 cells correlated with increased tyrosine phosphorylation of $p185^{HER2}$ and its association with Shc molecule, with the recruitment of Grb-2 molecules into complexes, and with the tyrosine phosphorylation of MAPK proteins, indicating that MGr6-stimulated $p185^{HER2}$ triggers the Shc/Grb-2/MAPK pathway normally used by the receptor. Together, these results indicate that $p185^{HER2}$ overexpression per se is ineffective in triggering $p185^{HER2}$ cellular signaling and that binding-dependent activation of $p185^{HER2}$ is essential in inducing PC12 cell differentiation indistinguishable from that induced by NGF.

The identification of a ligand or a peptide able to directly regulate the $p185^{HER2}$ activity

might be useful in therapeutic approaches aimed at inducing differentiation and cell growth arrest in tumors overexpressing p185$^{HER2}$. The ability of PC12–HER2 cells to differentiate only after binding-dependent p185$^{HER2}$ activation and the rapid scoring of differentiation using light microscope analysis make this cell line a powerful tool in functional screening for activating ligands, such as peptides, phage-displayed peptides or antibody fragments that can bind the extracellular domain of p185$^{HER2}$, or for specific inhibitors of p185$^{HER2}$ tyrosine kinase activity.

## ACKNOWLEDGMENTS

This work was partially supported by the PF ACRO-CNR and the AIRC/FIRC. We thank P.P. Di Fiore for providing the LTR-2/erbB-2 plasmid and NIH/3T3–erbB-2 cells and M.G. Borrello for advice and reagents used in immunoprecipitation experiments. We also thank Piera Aiello for antibody purification, Mario Azzini for graphics work, and L. Mameli and D. Labadini for secretarial help.

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