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Involvement of cdc2-mediated phosphorylation in the cell cycle-dependent regulation of p185^{neu}

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We previously reported cell cycle-dependent negative regulation of p185^{neu} (decreased tyrosine phosphorylation and kinase activity, with electrophoretic mobility retarded by serine/threonine phosphorylation) in M phase and the escape of mutation-activated p185^{neu*} from this regulation. Our present results showed that retardation of electrophoretic mobility occurs independently of the cells' transformed status. We found that normal p185^{neu} lost its ability to dimerize in the M phase. We demonstrated a physical association between cdc2 (a serine/threonine kinase, active in M phase) and p185^{neu}. We showed that the carboxy terminal portion of p185^{neu} is phosphorylated *in vitro* by cdc2. Many phosphopeptides (at least three phosphoserine residues) unique to the M phase were identified, and the *in vivo* and *in vitro* phosphopeptide patterns were superimposable. In contrast, mutation-activated p185^{neu*} dimerized in the M phase with no changes in electrophoretic mobility, failed to associate with cdc2 and no unique phosphoserine residues could be identified in the M phase (data not shown), consistent with the escape of p185^{neu*} from cell cycle-dependent regulation. Our results suggest that this escape is an intrinsic property of the mutation-activated p185^{neu*} independent of its ability to transform cells. Our results also suggest the involvement of serine/threonine kinases such as cdc2 in the cell cycle-dependent negative regulation of p185^{neu}.

Keywords: cdc2; cell cycle; p185^{neu}; phosphoamino acid analysis; peptide mapping; serine/threonine kinases

Introduction

The HER2/*neu*/c-*erb*B-2 gene encodes a 185 kDa transmembrane glycoprotein (p185^{neu}) that belongs to the EGF receptor family (Bargmann *et al*., 1986a,b; Bargmann and Weinberg, 1988a,b; Hung *et al*., 1986; Padhy *et al*., 1982; Schechter *et al*., 1984). Members of this family are characterized by an extracellular, transmembrane, and the cytoplasmic tyrosine kinase domains. While the extracellular domain is involved in ligand binding, the cytoplasmic carboxy terminal tails display sequence heterogeneity and carry several tyrosine (including the autophosphorylation sites), serine, and threonine residues (Yarden and Ullrich, 1988). In many cellular systems, both dimerization and tyrosine kinase activation follow second order kinetics with respect to receptor concentration, which implicates dimerization as an activating event for the kinase domain and suggests that the dimers are important structures in the propagation of mitogenic signals from the cell surface to the nucleus (Heldin, 1995).

It is known that a single-point mutation in the transmembrane region (Val-644 to Glu) of *neu* converts the *neu* protooncogene into a transforming *neu* oncogene (*neu**) (Bargmann *et al*., 1986a; Bargmann and Weinberg, 1988b; Hung *et al*., 1989; Muller *et al*., 1988; Stern *et al*., 1988). The mutation-activated p185^{neu*} is associated with higher tyrosine kinase activity and exhibits more potent transforming ability than normal p185^{neu} (Bargmann and Weinberg, 1988a; Stern *et al*., 1988; Weiner *et al*., 1989). Although a direct ligand of p185^{neu} is still not known, p185^{neu*} exists predominantly in the dimer form, and p185^{neu} exists as a monomer (Weiner *et al*., 1989). Indeed, theoretical (Brandt-Rauf *et al*., 1990; Smith and Bormann, 1996; Sternberg and Gullick, 1990) and experimental evidence (Weiner *et al*., 1989) implied that the oncogenic mutation maintains the receptor in a dimer form that is analogous to the ligand-induced dimer of the EGF receptor (Yarden and Schlessinger, 1987a,b). By dimerizing the receptor, the oncogenic mutation seems to establish a high affinity binding state that is functionally equivalent to that of the ligand-occupied normal receptor (Ben-Levy *et al*., 1992). We demonstrated that the structure and function of p185^{neu} are regulated through the cell cycle. In M phase, p185^{neu} is least active in tyrosine phosphorylation function and the regulation may occur through the alteration of phosphorylation of serine and/or threonine residues. Point mutation allowed p185^{neu} tyrosine kinase to escape cell cycle regulation as it remained constitutively active throughout the cell cycle (Kiyokawa *et al*., 1995).

In the present study we sought further understanding of the mechanism of the cell cycle-dependent negative regulation of p185^{neu} we had shown. Using a cross-linking agent, we found that the normal p185^{neu} does not dimerize in the M phase, but that the oncogenic p185^{neu*} is dimerized. Further experiments with co-immunoprecipitation, *in vitro* kinase assays, and *in vivo* ^{32}P metabolic labeling methods, suggested the involvement of cdc2, an M phase-specific serine/threonine kinase, in the negative regulation.

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Results

Escape from cell cycle-dependent regulation is an intrinsic property of the mutation-activated p185^{neu*}, independent of its ability to transform cells

As shown earlier, the normal p185^{neu} protein is regulated in a cell cycle-dependent manner, whereas the p185^{neu*} (with a point mutation in the transmembrane region) escapes from this regulation. With cell-cycle progression, tyrosine phosphorylation and tyrosine kinase activity of p185^{neu} gradually decreased, reaching their minimum level in M phase. An overall hyperphosphorylation in the serine and/or threonine residues resulted in an electrophoretic mobility shift in the M phase. The oncogenic p185^{neu*} protein tyrosine kinase was constitutively active in both G_0/G_1 and M phases while the protein's electrophoretic mobility did not change significantly. Thus, the single-point mutation in the transmembrane domain facilitated the escape of p185^{neu*} from cell cycle dependent negative regulation of p185^{neu} tyrosine kinase activity, possibly as a result of serine/threonine phosphorylation (Kiyokawa *et al*., 1995). Since the single-point mutation also induced cellular transformation, it was pertinent to ask whether the absence of the mobility shift in M phase is a specific property of the mutated *neu** or is caused by the transformation *per se*. To address this question, NIH3T3 cells (expressing endogenous p185^{neu}) transformed by different oncogenes (*ras*, *src* and *abl*) were synchronized either in G_0/G_1 or M phase and their electrophoretic mobility was analysed after their cell cycle stage was confirmed by DNA content analysis. As shown in the two upper panels of Figure 1, DNA content analysis confirmed that the serum starvation synchronized the parental NIH3T3 and the transfected cells in G_0/G_1 stage, and that nocodazole treatment arrested these cells in the M phase. The results, shown in the lower panel of Figure 1 depicting the electrophorectic mobility changes in p185^{neu} detected by Western blotting, indicate retarded migration of the protein in M phase in all cells analysed. Mutated p185^{neu*} did not show a change in electrophoretic mobility at different stages of the cell cycle (Kiyokawa *et al*., 1995) and the cells transformed by various oncogenes (*ras*, *src* and *abl*) also showed retarded electrophoretic mobility (Figure 1). These results suggested that the escape from cell cycle-dependent regulation is an intrinsic property of the mutation-activated p185^{neu*}, independent of its ability to transform cells.

Normal p185^{neu} fails to dimerize in M phase but not the mutation-activated p185^{neu*}

Since the function of p185^{neu} is tightly associated with its dimerization status (Weiner *et al*., 1989), we next asked whether this could account for the negative regulation of p185^{neu} in M phase and the escape of p185^{neu*} from this process. We used derivatives of NIH3T3 cells that express the normal *neu* (DHFR/G8) and the mutated *neu** (B104-1-1). After synchronizing these cells in G_0/G_1 or M phase, they were crosslinked and p185^{neu} proteins were analysed by Western blotting. As predicted, the DHFR/G8 cells failed to dimerize in the M phase, and the dimerization status remained unaltered in B104-1-1 cells between G_0/G_1 and M phases (Figure 2). Immunoprecipitation of p185^{neu} after crosslinking under the same conditions also gave similar results (data now shown).

Added to our previous report (Kiyokawa *et al*., 1995), these data suggested that, in M phase of the cell cycle, the serine/threonine hyperphosphorylation of p185^{neu} may prevent receptor dimerization, which in turn inhibits the tyrosine kinase activity of p185^{neu} and intrinsically aids the escape of p185^{neu*} from this regulation.

Physical association between cdc2 and p185^{neu}

Interestingly, cdc2, a serine/threonine kinase, is most active in M phase (Bischoff *et al*., 1990; Draetta and Beach, 1988; Lee and Nurse, 1987; Shenoy *et al*., 1989).

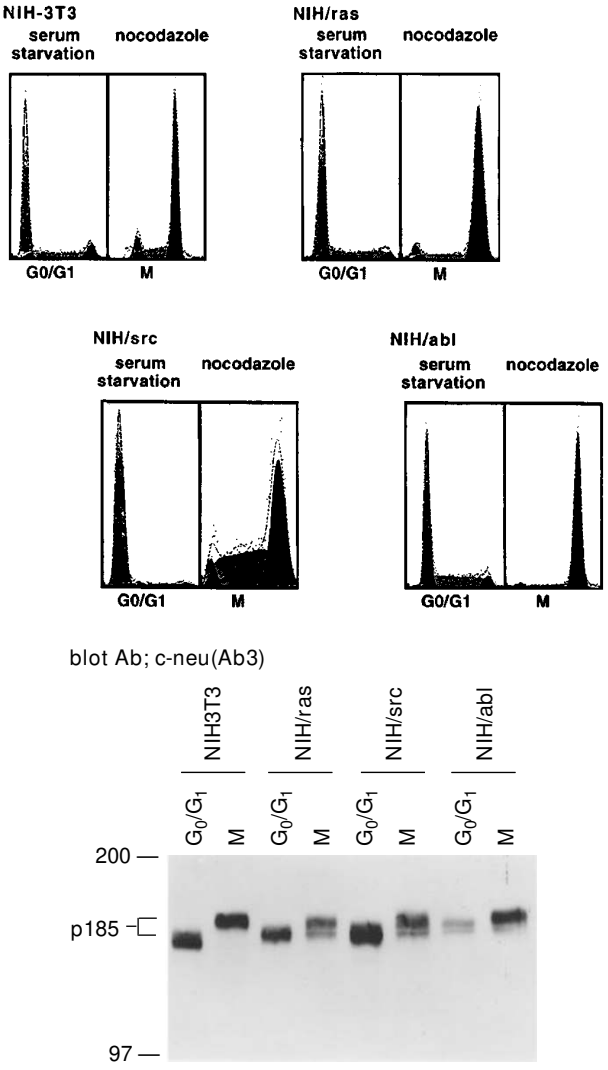

Figure 1 DNA content analysis and electrophoretic mobility changes in G_0/G_1 or M phase. Upper panel: NIH3T3, NIH/*ras*, NIH/*src*, and NIH/*abl* cells were either serum-starved (G_0/G_1) or treated nocodazole (M) and trypsinized for the DNA content analysis described in Materials and methods. Lower panel: Total lysates from either serum-starved (G_0/G_1) or nocodazole-treated (M) NIH3T3, NIH/*ras*, NIH/*src* and NIH/*abl* cells were analysed, after electrophoresis, by Western blotting with a monoclonal anti-p185^{neu} antibody. The experiments were repeated at least twice with similar results

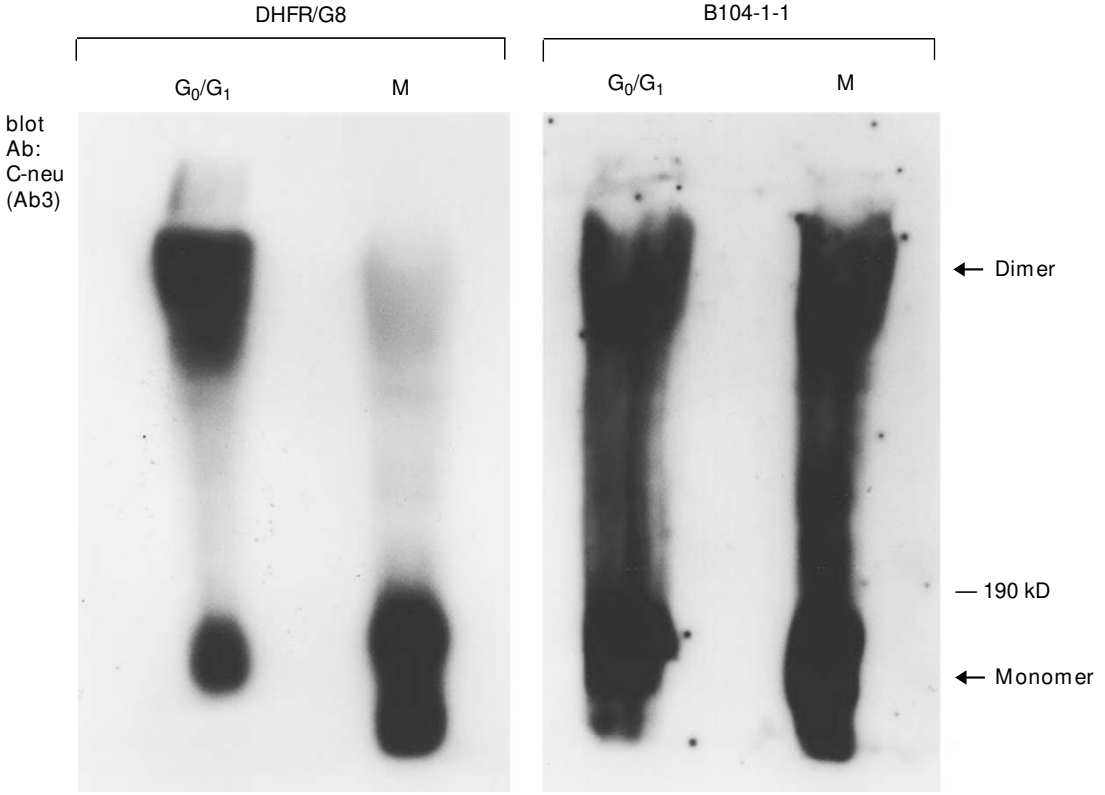

Figure 2 Dimerization in G_0/G_1 or M phase. DHFR/G8 and B104-1-1 cells were synchronized either in G_0/G_1 or M phase and then crosslinked with BS^3 as described in Materials and methods. After electrophoresis, the total lysates were analysed by Western blotting with a monoclonal anti-p185^{neu} antibody. The experiment was done twice with reproducible results

Perhaps not by coincidence, a careful examination of the amino acid sequence of p185^{neu} revealed no less than 17 potential sites for cdc2. To see whether the two different kinases, cdc2 and p185^{neu}, are physically associated, we immunoprecipitated the cell lysates of DHFR/G8 expressing normal p185^{neu} and B104-1-1 expressing p185^{neu*} with a monoclonal antibody to cdc2 or with normal mouse serum (NMS) and immunoblotted them with a monoclonal antibody to p185^{neu} or cdc2. The results shown in Figure 3a indicated that these two proteins are physically associated in DHFR/G8 cells, but not in B104-1-1 cells that express p185^{neu*}. These results were ascertained by using three different antibodies to immunoprecipitate cdc2 under the same experimental conditions (data not shown). We also examined the physical association between cdc2 and p185^{neu} in different stages of the cell cycle in DHFR/G8 cells, by chemical synchronization, and found relatively abundant association between them at G2/M phase (Figure 3b).

cdc2 can phosphorylate p185^{neu} in vitro

Encouraged by the physical association of cdc2 and p185^{neu} in DHFR/G8 cells, we used an *in vitro* complex kinase assay to investigate whether cdc2 can phosphorylate p185^{neu}. Phosphorylation of p185^{neu} by cdc2 was observed when the p185^{neu} was immunoprecipitated from the DHFR/G8 cells and then mixed with commercially available active cdc2, or when the cell lysates from DHFR/G8 cells were frozen and thawed a few times after immunoprecipitation (to reduce the autophosphorylation activity of p185^{neu} tyrosine kinase) and mixed (Figure 3c). Phosphorylation of p185^{neu} was not seen, however, when cdc2 or p185^{neu} was present alone in the reaction mixture (Figure 3c). The phosphorylated band of p185^{neu} from the above experiment was further examined by phosphoamino acid analysis, and the results confirmed the involvement of phosphorylation of serine and threonine but not of tyrosine residues (Figure 3d) which was predictable from the known action of cdc2 as a serine/threonine kinase. Using different antibodies to immunoprecipitate cdc2, we also confirmed that the immunoprecipitated p185^{neu} from DHFR/G8 cells could be phosphorylated by cdc2 *in vitro* (data not shown).

cdc2 phosphorylates the carboxy terminal of p185^{neu}

In an attempt to locate the phosphorylation sites of p185^{neu} in M phase by cdc2, we carried out an *in vitro* and *in vivo* two-dimensional phosphopeptide mapping analysis. The approximate positions of the potential phosphorylation sites for cdc2 in the p185^{neu} protein are schematically shown in Figure 4a. The drawing depicts GST-fusion protein that represents the carboxy terminal region of p185^{neu} (YX1) and contains most of the serine and threonine residues, and another GST protein (YX3) that contains a few serine and threonine residues constructed from the kinase domain and the juxtamembrane portion of p185^{neu}. The expression of these fusion proteins was revealed by coomassie blue staining of the bacterial cell lysates and the major

proteins of expected size were stained heavily by the dye and further confirmed by immunoblotting analysis, which showed that an antibody directed against the carboxy terminal tail of *neu* detected only YX1 and not YX3 (Figure 4b). Similarly, the use of an antibody against the kinase domain reacted with YX3 but not YX1, as expected (Figure 4b). These fusion proteins were then used in an *in vitro* complex kinase assay that utilized the commercially available purified cdc2 (UBI). The results showed that only YX1 made from the carboxy terminal of p185^{neu} was phosphorylated when mixed with cdc2, and that cdc2 : YX3 mix had no such effect (Figure 4c). Phosphorylation was not observed when either YX1 or YX3 was present in the reaction mixture without cdc2 (data not shown). Taken together, these results strongly suggested the existence of potential phosphorylation sites for cdc2 at the carboxy terminal tail of p185^{neu}.

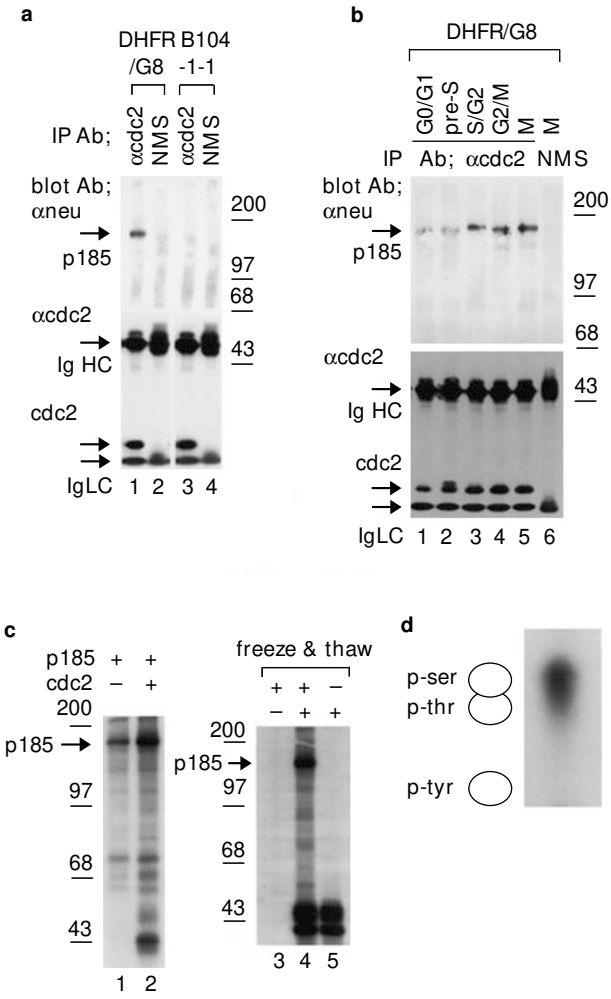

Figure 3 Physical association of normal p185^{neu} with, and its phosphorylation *in vitro* by, cdc2. (**a**) Co-immunoprecipitation of p185^{neu} with cdc2. The proteins were immunoprecipitated with a monoclonal anti-cdc2 antibody (α cdc2) or normal mouse serum (NMS) from 500 μg of precleared lysate of DHFR/G8 or B104-1-1 cells. After intensive washing with lysis buffer, samples were separated on 6–10% SDS–PAGE gradient gel and transferred onto a nitrocellulose membrane. The membrane was cut into two parts to detect both p185^{neu} and cdc2 by immunoblotting with either monoclonal anti-p185^{neu} antibody (c-*neu* (Ab3), upper panel) or monoclonal anti-cdc2 antibody (αcdc2, lower panel). (**b**) Cell cycle dependent association of p185^{neu} with cdc2. DHFR/G8 cells were synchronized at different stages of cell cycle as described in Materials and methods. Cell lysates were prepared, immunoprecipitated and analysed as described for a. (**c**) *In vitro* phosphorylation of p185^{neu} by cdc2. The ability to phosphorylate p185^{neu} was tested by an *in vitro* immune complex kinase assay. Immunoprecipitated p185^{neu} proteins from DHFR/G8 were equally divided into two parts and mixed with (+) or without (−) the purified cdc2 protein (25 ng/assay) followed by incubation with [γ-^{32}P]ATP and $MgCl_2$ for 20 min at room temperature. After separation on 7% SDS–PAGE gel samples were exposed on X-ray film. To minimize autophosphorylation activity, proteins denatured by repeated freezing and thawing were tested in a similar manner. (**d**) Phosphoamino acid analysis. Phosphorylated p185^{neu} proteins from lane 4 of **c** were excised and phosphoamino acid analysis was performed as described in Materials and methods. Ig HC-Immunoglobulin heavy chain; Ig LC-Immunoglobulin light chain. The results from **a**, **b**, **c** and **d** were confirmed at least in another independent experiment

In vitro *and* in vivo *phosphopeptide patterns are superimposable*

The possible relevance of cdc2-mediated phosphorylation of p185^{neu} observed *in vitro* was next examined *in vivo* by metabolic labeling of DHFR/G8 cells with ^{32}P before (unsynchronized) or after the cells were synchronized in G_0/G_1 or M phase and their phosphopeptide pattern analysed, as shown in Figure 5. When phosphopeptides from G_0/G_1 and M phase were mixed together and analysed, more than nine phosphopeptides unique to the M phase could be identified, along with two phosphopeptides unique to the G_0/G_1 phase, and several phosphopeptides common to both G_0/G_1 and M phases. Phosphoamino acid analysis of the phosphopeptides revealed that at least three phosphopeptides unique to the M phase contained phosphorylated serine residues, and several such residues were found also in the phosphopeptides common to both phases of the cell cycle (Figure 5). When a similar experiment was done with B104-1-1 cells, the phosphopeptide patterns of G_0/G_1 and M phases were virtually identical (notably, they resembled the G_0/G_1 stage of DHFR/G8 cells), and no unique phosphoserine residues could be identified in the M phase (data not shown), consistent with the escape of p185^{neu*} from cell cycle-dependent regulation. Thus, our results suggested that cdc2 can phosphorylate p185^{neu} in M phase in DHFR/G8 cells. If this is indeed the case, the *in vitro* and *in vivo* phosphopeptide mapping patterns should be similar. When the phosphopeptides obtained from the YX1-cdc2 complex (Figure 4c) were mixed with the *in vivo* -labeled phosphopeptides prepared from the M phase synchronized DHFR/G8 cells and analysed, we found more than 10 common phosphopeptide spots among the *in vivo* and *in vitro* patterns (Figure 6). Phosphoamino acid analysis helped us to identify at least five phosphoserine-containing phosphopeptides among them, supporting the involvement of cdc2. The presence of several such peptides unique to the M phase (Figure 6) indicated that other serine/threonine kinases could also be involved in the negative regulation of p185^{neu}.

Discussion

We pursued our earlier report which showed the disruption of the cell-cycle dependent regulation of normal p185^{neu} by its oncogenic counterpart p185^{neu*} and defined a plausible mechanism of cellular

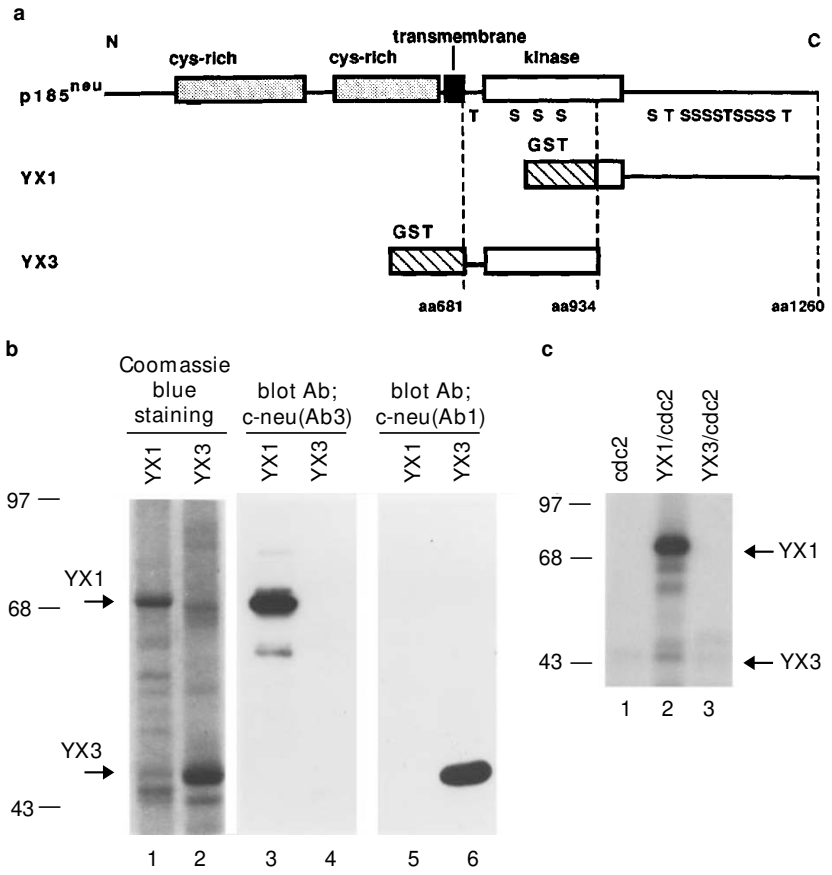

Figure 4 *In vitro* phosphorylation of recombinant p185^{neu} protein of cdc2. (**a**) Construction of GST-fusion p185^{neu} proteins are shown by the schema. (**b**) Recombinant-GST-fusion p185^{neu} proteins were purified as described in Materials and methods, and their molecular weights were examined by SDS–PAGE followed by coomassie blue staining (lanes 1 and 2). Both proteins (YX1 and YX3) were immunoblotted with either anti-p185^{neu} c-terminal antibody (c-*neu* Ab-3; lanes 3 and 4) or anti-p185^{neu} kinase domain antibody (c-*neu* Ab-1; lanes 5 and 6). (**c**) Purified cdc2 protein (25 ng/assay) was incubated with YX1 (lane 2) or YX3 (lane 3) or without the recombinant proteins (lane 1) in the presence of [γ-^{32}P]ATP and $MgCl_2$. The ability of cdc2 to phosphorylate the fusion proteins was examined as described in Figure 3. Similar results were obtained when the experiment was repeated two times

transformation (Kiyokawa *et al*., 1995). The present results clarified that the disruption is not caused by the transformation *per se* (Figure 1), but that the escape of p185^{neu*} from regulation may be an intrinsic property of the mutated protein. We had predicted that the negative regulation of normal p185^{neu} tyrosine kinase activity observed in M phase of the cell cycle in DHFR/G8 cells and an escape from this regulation in B104-1-1 cells that express p185^{neu*} (Kiyokawa *et al*., 1995) could be the result of a disequilibrium between the p185^{neu} receptor's monomeric and dimeric forms. The results shown in Figure 2 lend strong support to this prediction because the dimers could not be captured by crosslinking during M phase in DHFR/G8 cells. Weiner *et al*. (1989) have analysed the formation of dimers in detail in DHFR/G8 and B104-1-1 cells. They found that about 70% of the oncogenic p185^{neu*} exists as an aggregated cross-linkable complex, and 20–30% of normal p185^{neu} in DHFR/G8 cells can be cross-linked under identical conditions. It is interesting to note that, in a family of bacterial receptors that have the same topology as the eukaryotic single-transmembrane receptors, general structural changes (such as a monomer-to-dimer transition) seem to have been responsible for transmembrane signaling rather than specific ligand-induced conformational changes (Stock, 1996). Ligand-independent dimerization is known to occur in oncogenic tyrosine and serine/threonine receptor kinases (Adelsman *et al*., 1996; Carlberg and Rohrschneider, 1994; Vivien *et al*., 1995; Watowich *et al*., 1992; Weiner *et al*., 1989).

More intriguing is the mechanism by which such negative regulation results in the failure to dimerize (Figure 2) and the consequent decrease in tyrosine kinase activity and signaling of the normal p185^{neu} receptor (Kiyokawa *et al*., 1995) in M phase. We hypothesized that it is due to the hyperphosphorylation of serine/threonine residues in M phase and the amino acid mutation in p185^{neu*} disrupts this regulation. The presence of more phosphopeptide spots (at least 3 containing phosphoserine residues) in the M phase than in the G_0/G_1 phase (which had no phosphoserine residues) of DHFR/G8 cells that express normal p185^{neu} (Figure 5), and identical phosphopeptide patterns of G_0/G_1 and M phase-synchronized B104-1-1 cells that express mutated p185^{neu*} (data not shown), also support this possibility. The presence of 17 cdc2 consensus sites in the amino acid sequence of p185^{neu} and cdc2's role as a serine/threonine kinase most active in M phase (Bischoff *et al*., 1990; Draetta and Beach, 1988; Lee and Nurse, 1987; Shenoy *et al*., 1989)

qualifies it as a potential candidate for the described negative regulation. Consistent with these structural features and M phase-specific activity are our experimental results (Figure 3a and b) which show that physical association between these two proteins is stronger at G2/M phase of the cell cycle. Though cdc2 is a nuclear protein, its existence and activation in the cytoplasm has also been reported (Heald *et al*., 1993). Our results demonstrated cdc2 site of action to be the carboxy terminal of p185^{neu} (Figure 4) after showing cdc2 to be capable of phosphorylating p185^{neu} *in vitro* and the phosphorylation sites to overlap with those *in vivo* (Figures 3 and 5). Furthermore, the superimposable phosphopeptide patterns obtained when *in vitro* cdc2-phosphorylated YX1 (carboxy-terminal p185^{neu} -GST fusion protein) was mixed with the *in vivo* labeled M phase-synchronized DHFR/G8 cells (Figure 6) strongly suggested the involvement of cdc2. Further work is needed to locate specific serine/threonine residues in p185^{neu} phosphorylated by cdc2. Mutation of such residues would reveal their relative functional importance, though the question is complicated by the presence of several serine/threonine residues. Purified *Xenopus laevis* p34cdc2 was reported to phosphorylate the EGFR on serine 1002 *in vitro*, implying that receptor function may be regulated in a cell cycle-dependent fashion (Kuppuswamy *et al*., 1993). In addition, the *in vitro* phosphorylation was associated with an inhibition of EGFR tyrosine kinase activity. A comparison of the sequences of several growth factor receptors showed that the string of acidic residues, followed by the Ser-Pro motif found at residues 1002–1003 of the EGFR, a proposed cdc2 phosphorylation site, is conserved in most EGFR family members, including p185^{neu} (Bargmann *et al*., 1986a), ErbB-3 (Plowman *et al*., 1990), and let-23, the EGFR homologue in *C. elegans* (Aroian *et al*., 1990). However, a role for these cell cycle stage-specific phosphorylations remains an intriguing possibility in the negative regulation of the receptor tyrosine kinases, including the EGFR and p185^{neu}. A better known physiological feedback-inhibitory mechanism involves protein kinase C (PKC), a serine/threonine kinase, which phosphorylates threonine 654 residue on the EGFR, decreasing the receptor's binding affinity for ligands (Morrison *et al*., 1993), diminishing tyrosine kinase activity (Cochet *et al*., 1984; Davis, 1988), and attenuating signals from the EGFR (Chen *et al*., 1996).

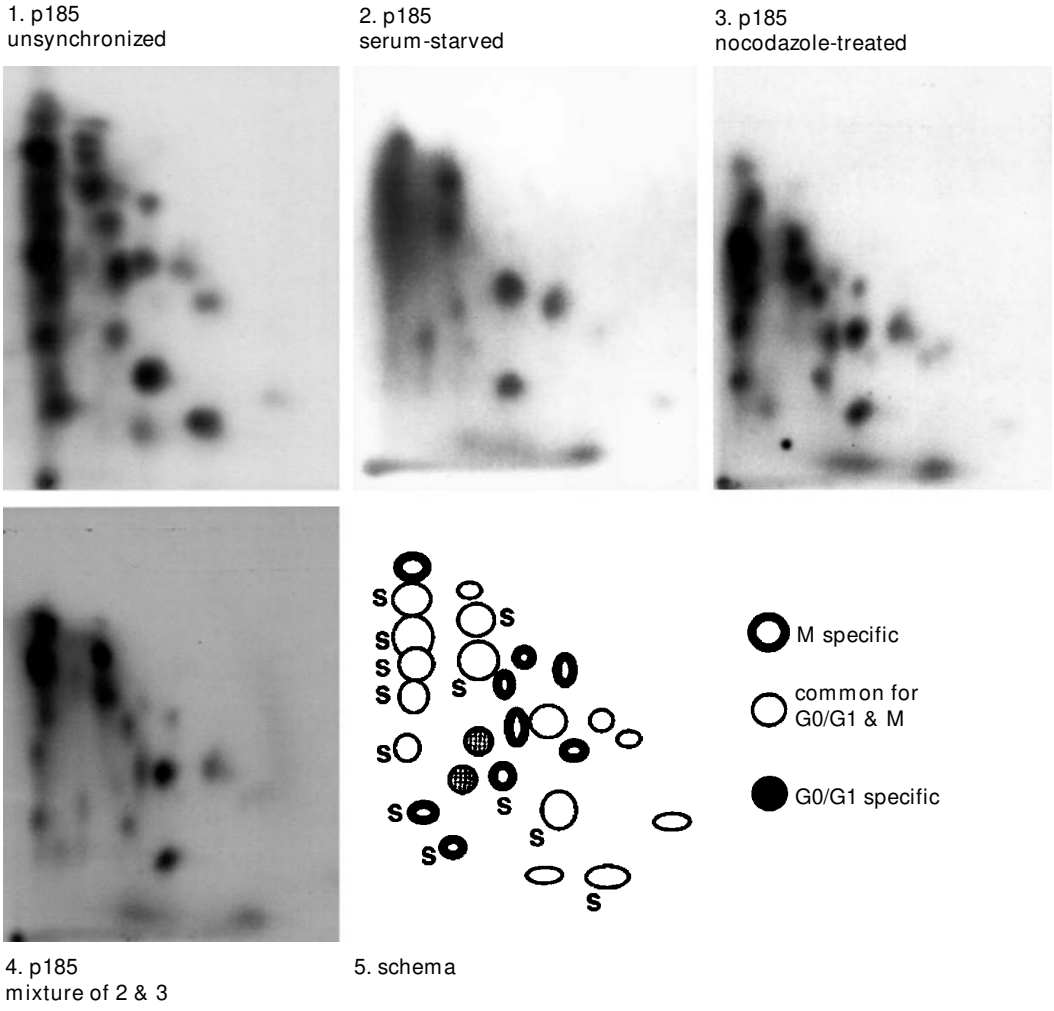

Figure 5 Phosphopeptide analysis of metabolically labeled p185^{neu} in G_0/G_1 or M phase. The p185^{neu} proteins from DHFR/G8, metabolically labeled with γ-^{32}P from unsynchronized (panel 1), serum-starved (panel 2), or nocodazole-treated (panel 3) cells were purified by immunoprecipitation, and the individual samples or an equal mixture from panel 2 and 3 (panel 4) were separated by SDS–PAGE gel and analysed for the presence of phosphopeptides as described in the Materials and methods. Individual phosphopeptide spots from the panel 3 were cut out, and phosphoamino acid analysis identified several phosphoserine residues (S), as shown in schema (panel 5). Two independent experiments were done to confirm these results

Feedback inhibition of p185^{neu} tyrosine kinase activity by PKC-mediated serine/threonine phosphorylation has also been reported (Cao *et al*., 1991; Dobashi *et al*., 1989). Although our results suggested the involvement of cdc2 in the phosphorylation of p185^{neu}, they do not rule out the involvement of other kinases in the process. The presence of additional phosphopeptides, several of them containing phosphoserine residues, in *in vivo* -labeled DHFR/G8 cells in M phase (Figure 6) already indicates this possibility. HER2/*neu* is phosphorylated *in vitro* on a single site in the cytoplasmic tail at threonine 1172 by the calmodulin-dependent protein kinase II (Feinmesser *et al*., 1996). The consensus sites of cdc2 (basic-Ser/Thr-Pro-polar-basic or basic/polar-Ser/Thr-Pro-X-basic) are, in fact, quite similar to those of MAP kinase (Pro-X-Ser/Thr-Pro), another Ser/Thr kinase, where X represents any amino acid present in proteins (Langan *et al*., 1989; Lin *et al*., 1991; Shenoy *et al*., 1989). In addition, the platelet-derived growth factor receptor (Yarden *et al*., 1986) and fibroblast growth factor receptor (Lee *et al*., 1989) also contain Ser-Pro motifs in the region C terminal to the kinase domain, although they lack the acidic region upstream from this sequence. Serine/threonine phosphorylation is likely to be a general phenomenon since a number of growth factor receptors could be phosphorylated by a proline-directed kinase in this region. It would be interesting to analyse the action of various serine/threonine kinases and their involvement in and relative contribution to negative regulation of receptor tyrosine kinases in general and the cell cycle in particular.

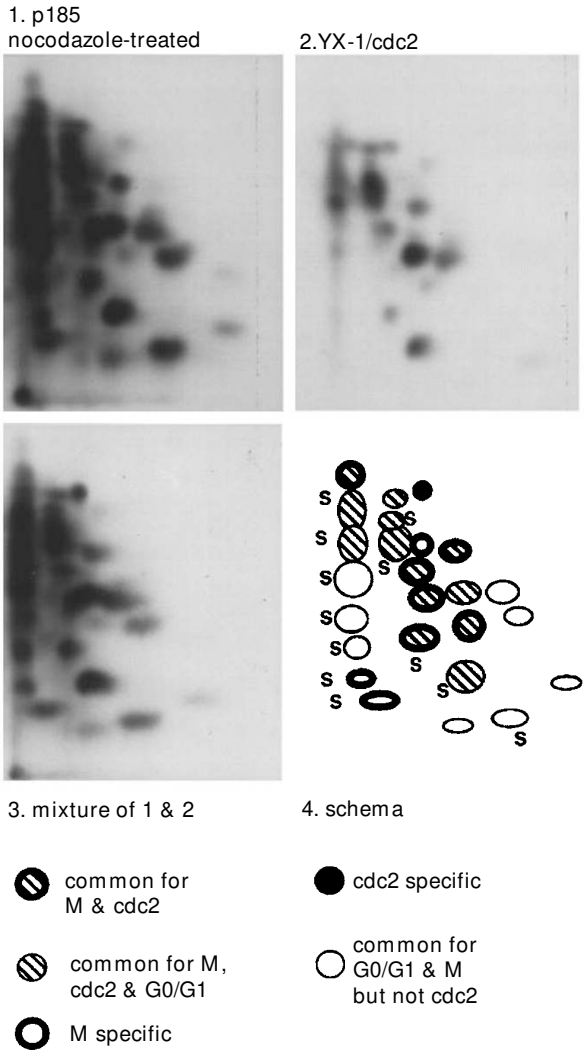

Figure 6 Comparison of the patterns of phosphopeptides and phosphoamino acids obtained from the *in vitro* and *in vivo* phosphorylation of p185^{neu} protein. DHFR/G8 cells were metabolically labeled after treatment with nocodazole and analysed as described in Figure 5 and the phosphopeptide spots are shown in panel 1. The recombinant-GS-p185^{neu} fusion protein (YX-1) was phosphorylated and analysed as described in Figure 4 and shown in panel 2. The phosphopeptide pattern obtained after mixing the samples from panels 1 and 2 are shown in panel 3. Phosphoamino acid analysis of individual spots from panel 3 revealed the prescence of several phosphoserine-containing peptides (S) as shown in the schema (panel 4). These results were confirmed in another independent experiment

Materials and methods

Cell culture and synchronization

Cell lines that express the normal p185^{neu} (DHFR/G8) and the mutation-activated p185^{neu*} have been described (Hung *et al*., 1986; Kiyokawa *et al*., 1995; Padhy *et al*., 1982). NIH3T3 cells were transfected with *ras*, *src* and *abl* oncogenes (NIH/*ras*, NIH/*src*, NIH/*abl*) as described earlier (Matin and Hung, 1994). Cells were grown in Dulbecco's modified Eagle medium : nutrient mixture F-12 (DMEM/F12 1 : 1) (GIBCO) supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO_2 atmosphere. Cells at half-confluence were starved in DMEM/F12 with 0.5% FCS for 48 h to arrest them in G_0/G_1 phase (Welch and Wang, 1993). To accumulate in pre-S phase and G2 phase, cells were incubated with aphidicolin and Hoechst 33342, respectively, as described (Kiyokawa *et al*., 1995). To synchronize the cells in M phase, they were incubated with DMEM/F12 containing 10% fetal calf serum and nocodazole (0.4 μg/ml) for 12–24 h, afterwhich cells that had a highly rounded mitotic shape were collected by mechanical shake-off. More than 90% of the nocodazole-treated shaken off cells were tetraploid according to DNA content analysis (Chackalaparampil and Shalloway, 1988). The adherent cells after nocodazole treatment were considered to be in G2/M phase.

DNA content analysis

For DNA content analysis, trypsinized cells were fixed for 30 min at −20°C in 70% ethanol-30% PBS mixture. After staining with PBS containing 5 μg/ml propidium iodide and 8 μg/ml RNAse A, cells were analysed on an EPICS PROFILE flow cytometer (Coulter) as described previously (Chackalaparampil and Shalloway, 1988). The results were analysed with the MultiCycle computer program (Pheonix Flow System).

Antibodies

Anti-cdc2 monoclonal antibody p34 (Santa Cruz Biotechnology Inc.,) was used for both immunoprecipitation and immunoblotting. Anti-p185^{neu} monoclonal antibody raised against the c-terminal (Ab-3) or the extracellular portion (Ab-4) and a rabbit antibody raised against the kinase domain (Ab-1) were obtained from Oncogene Science.

Generation of GST-p185^{neu} fusion proteins

cDNA fragments that correspond to different parts of the intracellular portion of rat p185^{neu} were amplified by PCR from pSV2neu and subcloned into pGEX-3X bacterial

expression vector (Pharmacia) at *Hind*III and *Sma*I sites. The GST-fusion proteins, YX-1 representing amino acids 934–1260 and YX-3 representing amino acids 681–993 of p185^{neu}, were produced in bacteria and purified.

Immunoprecipitation and Western blotting

After each treatment, cells were solubilized for 30 min on ice in lysis buffer (20 mM Na_2PO_4, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 100 mM NaF and 2 mM Na_3VO_4) and the total protein concentration was determined using the Bio-Rad protein assay kit. Electrophoretically separated proteins were transferred to a nitrocellulose membrane for 1 h at 3 mA/cm^2 using a semidry transblot system (Bio-Rad). After being blocked with PBS containing 5% milk, membranes were incubated with primary antibodies for 1 h at room temperature in 3% BSA/PBS, then washed with 0.05% Tween 20 in PBS. After 30 min of incubation with an appropriate secondary antibody conjugated with horseradish peroxidase and a subsequent washing with PBS-0.05% Tween 20, immunoblots were developed by the enhanced chemiluminescence reagent (ECL, Amersham) for 1 min and exposed to film (Hyper-film, Amersham). For immunoprecipitations, 500 μg to 1 mg of cell lysates were incubated with 1 μg of antibody and 50 μl of 50% protein-A agarose for 1 h at 4°C. After several washes with 0.05% Tween 20/PBS, immunoprecipitates were separated by electrophoresis and analysed.

Chemical crosslinking

Cells synchronized in the G_0/G_1 or M phase were washed with PBS twice and incubated with 1 mM bis(sulfosuccinimidyl) suberate (BS^3) in PBS for 30 min at 4°C, with rocking. The cells were solubilized with PI/RIPA buffer (Weiner *et al*., 1989).

In Vitro *immune complex kinase assay*

Commercially available cdc2 complex (Upstate Biotechnology Incorporated, New York) purified from the sea star *Pisaster ochraceus* was mixed with either the immunoprecipitated p185^{neu} proteins or the recombinant GST-p185^{neu} proteins. After being washed four times, once with PBS, twice with 50 mM Tris-HCl (pH 7.5) containing 0.5 M LiCl and once with kinase assay buffer (50 mM Tris, 10 mM $MgCl_2$, pH 7.5), the immunoprecipitates were incubated for 20 min at room temperature in 50 μl of kinase buffer, with 10 μCi of [^{32}P]γ-ATP (specific activity >3000 Ci/mM; Amersham). Reactions were stopped by adding 10 μl of 6×SDS-sample loading buffer. After separation on a 7% SDS–PAGE gel, phosphorylated proteins were visualized with autoradiography.

^{32}P metabolic labeling

Cells were metabolically labeled with ^{32}P-orthophosphate (500 μCi/ml, carrier-free; Amersham) for 8 h in phosphate-free DMEM supplemented with either 0.5% or 10% dialyzed FCS (Epstein *et al*., 1990).

Phosphopeptide and phosphoamino acid analysis

In vitro or *in vivo* ^{32}P-labeled p185^{neu} proteins were excised and extracted from SDS–PAGE gel as described previously (Cooper *et al*., 1983). One portion was digested with TPCK-treated trypsin and the peptides were separated on TLC plates by electrophoresis (horizontal direction, anode on left) in pH 3.5 buffer and chromatography (vertical direction) as described previously (Kiyokawa *et al*., 1995). Another portion was hydrolyzed in 6 N constantly boiling HCL for 60 min at 110°C and separated on TLC plates with unlabeled phosphoamino acid standards by two-dimensional electrophoresis for phosphoamino acid analysis. In both cases, phosphopeptides and phosphoamino acids were detected by autoradiography and the positions of phosphoamino acids were visualized by ninhydrin staining.

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