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Synthetic phosphopeptide immunogens yield activation-specific antibodies to the c-erbB-2 receptor

(tyrosine kinases/oncogenes/phosphorylation/breast neoplasms)

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ABSTRACT **We inoculated rabbits with synthetic phosphopeptides, duplicating a major autophosphorylation site of the c-*erbB-2* protooncogene product. The rabbits produced antisera that, after reverse immunoaffinity purification, selectively recognize the erbB-2 protein in its enzymatically active configuration. These anti-phosphopeptide antisera identify a subset of erbB-2-positive human cell lines wherein the protein is constitutively active as a tyrosine kinase. Synthetic phosphopeptides incorporating informative protein phosphorylation sites may prove useful for generating antibodies that indicate the activation state of additional tyrosine kinases and perhaps other proteins phosphorylated on serine and threonine residues.**

The c-*erbB-2* (*neu* or *HER-2*) gene product is a cell-surface receptor tyrosine kinase structurally related to the epidermal growth factor (EGF) receptor. Clinical studies have established a link between tumor-associated c-erbB-2 protein overexpression and reduced survival of primary breast cancer patients with metastatic involvement of axillary lymph nodes (1–3). However, the association between c-erbB-2 protein expression and human breast cancer is neither pervasive nor internally consistent. Overexpression of c-erbB-2 in human breast tumors occurs more frequently in preinvasive than invasive lesions (4–6) and is seldom if ever associated with receptor-activating transmembrane domain mutations (7). In lymph-node-negative disease—where the need for a reliable prognostic indicator is especially pressing—correlations between expression of c-erbB-2 protein and clinical outcome are doubtful (1, 3, 8–10).

Since only the activated form of a tyrosine kinase is likely to influence cell growth and differentiation, these discrepancies could reflect heterogeneity of receptor activation *in vivo*. Patients with tumors expressing activated c-erbB-2 (due to mutations or paracrine/autocrine ligand production, for example) could prove to have a natural history different from that of patients whose tumors express predominantly inactivated receptor. To explore this possibility, we have developed antibodies that distinguish the kinase-active form of c-erbB-2 from the inert kinase-inactive configuration. Our approach is based on the use of synthetic phosphopeptide immunogens that duplicate a major c-erbB-2 receptor autophosphorylation site.

MATERIALS AND METHODS

Cells and Cell Lysis. Stock cultures of G8/DHFR murine fibroblasts containing an amplified dicistronic rat c-*neu*–dihydrofolate reductase construct (a gift of Robert Weinberg, Whitehead Institute, Cambridge, MA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) bovine calf serum, glutamine (60 mg/liter), penicillin (10 units/ml), streptomycin (0.1 μg/ml), and 0.3 μM methotrexate. All other cell lines were obtained from the American Type Culture Collection. Cell manipulation, immunoblot protocols, polyacrylamide gel electrophoresis, and reagents were as described (11). Cells were lysed in lysis buffer (LB) consisting of 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 150 mM NaCl, 1% Nonidet P-40, 10% (vol/vol) glycerol, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, protease inhibitors [40 μM leupeptin, aprotinin (10 μg/ml), and 1 mM phenylmethylsulfonyl fluoride], and tyrosine phosphatase inhibitors [1 mM sodium orthovanadate, benzamidine hydrochloride (50 μg/ml), and sodium molybdate (50 μg/ml)]. Approximately 250 μg of total protein lysate was added to each well for immunoblot analysis. To provide c-erbB-2-inactive controls, G8/DHFR cell samples were treated with 10% bovine calf serum for 15 min to transmodulate the c-erbB-2 receptor (11) and then lysed in the absence of tyrosine phosphatase inhibitors; the transmodulating efficiency of bovine calf serum is batch-dependent, probably reflecting varying platelet-derived growth factor content (R.J.E., unpublished observations). For immunoblot controls, pAb-1 rabbit polyclonal antibody (pan-erbB-2), raised against the unphosphorylated C-terminal (residues 1243–1255) peptide sequence of the *neu* gene product (Triton Biosciences, Alameda, CA), was used at a 1:100 dilution in TBST buffer (10 mM Tris·HCl, pH 8.0/150 mM NaCl/0.05% Tween 20), and monoclonal phosphotyrosine [Tyr(*P*)] antibody [anti-Tyr(*P*)] was purified over a *Staphylococcus aureus* protein A affinity column and used at a 1:1000 dilution. Immunoprecipitation of the c-erbB-2 receptor was performed using a 1:10 dilution of the monoclonal antibody mAb-1 (Triton Biosciences), which recognizes an extracellular domain receptor epitope.

***In Vitro* Kinase Assays and *in Vivo* Phospholabeling.** All manipulations were carried out at 4°C. Cell samples were lysed in LB and immunoprecipitated as described (11). The immunoprecipitates were washed twice with LB, twice with distilled H_2O, and twice with tyrosine kinase buffer (KB = 10 mM $MnCl_2$/20 mM Tris·HCl, pH 7.4). The beads were then drained, resuspended in 40 μl of kinase buffer containing 0.5 μl of [γ-^{32}P]ATP (specific activity, 3000 Ci/mol; 1 Ci = 37 GBq), and incubated at 4°C for 20 min. The reaction was stopped by addition of 1 ml of LB. The beads were washed once, resuspended in 50 μl of sample buffer, boiled for 5 min, and loaded onto a 7.5% polyacrylamide gel. After fixation and drying, the gel was exposed and analyzed using PhosphorImager model 400S; band quantification was carried out using IMAGEQUANT Version 2.0 software (Molecular Dynam-

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Abbreviations: EGF, epidermal growth factor; APHID, activation-specific phosphoprotein immunodetection; Tyr(*P*), phosphotyrosine; RIP, reverse immunoaffinity purification; apt, anti-phosphopeptide.

ics, Sunnyvale, CA). *In vivo* phospholabeling was carried out as described (11).

Synthesis of Tyrosine-Phosphorylated Peptides and Immunization Protocol. A major autophosphorylation site of the c-erbB-2 receptor expressed in NIH 3T3 cells is Tyr-1248 (12, 13). The C-terminal (residues 1243–1255) c-erbB-2 peptide sequence was synthesized in association with an N-terminal cysteine. Instead of incorporating a tyrosine residue at position 1248, however, a phosphorylated peptide was synthesized by standard Merrifield solid-phase synthesis procedures using the *t*-butoxycarbonyl strategy. Synthesis was performed on an ABI model 430 peptide synthesizer using small-scale (0.1 mmol) rapid cycles. Tyr(*P*) was incorporated as *tert*-butoxycarbonyl-*O*-(dibenzyl)phosphono-L-tyrosine (Peninsula Laboratories, Belmont, CA). The fully protected peptide was deprotected and cleaved from the phenylacetamidomethyl resin using trifluoromethanesulfonic acid. Reverse-phase analysis of the crude peptide was achieved on a Vydac C_{18} 5-μm column 250 mm × 2.1 mm. A linear gradient from 0 to 60 min was run with 0.1% trifluoroacetic acid and 0.09% trifluoroacetic acid in acetonitrile. Amino acid analysis was performed using standard Pico-Tag procedures (Waters). Hydrolysis was carried out with 6 M HCl at 145°C for 2 h. Composition analysis showed an almost complete conversion of Tyr(*P*) to tyrosine due to the hydrolysis condition. To show that Tyr(*P*) was indeed incorporated into the peptide, sequence analysis was performed using an ABI model 477 gas-phase sequencer. The presence of a Tyr(*P*) residue was determined by the lack of signal at the expected cycle [since Tyr(*P*) is not extracted by the solvents used] and the reemergence of the expected sequence on subsequent cycles. Phosphotyramine was prepared as described (14) and linked to Affi-Gel 10 beads (Bio-Rad). Amino acid analysis of Tyr(*P*)-1248-containing peptides revealed minimal unphosphorylated tyrosine. Preincubation of anti-Tyr(*P*) with the immunogen antagonized anti-Tyr(*P*) immunoprecipitation of B104-1-1 cell lysates containing activated c-erbB-2 (15) at least as efficiently as did 1 mM Tyr(*P*) (R.J.E., unpublished data). Cysteine-linked peptides corresponding to the C-terminal-domain c-erbB-2 peptide sequence (residues 1243–1255) and containing phosphorylated Tyr-1248 were coupled to activated keyhole limpet hemocyanin (Pierce). Rabbits were inoculated at ≈4-week intervals, and antisera were collected in 20- to 40-ml aliquots beginning 1 week after the booster injection.

Reverse Immunoaffinity Purification (RIP) of Antibody. RIP of antibody mixtures involves adsorbing unwanted antibodies to antigen(s) coupled to a solid phase (usually chromatography beads) and either eluting the remaining antibodies over the coupled antigen or removing the beads by centrifugation. RIP of anti-phosphopeptide antisera was undertaken by first coupling a nonphosphorylated cysteine-linked peptide (residues 1243–1255) to Affi-Gel 501 chromatography beads (Bio-Rad) for 16 h at 4°C. Beads were cleared of unbound peptide by six cycles of gentle centrifugation followed by resuspension in distilled H_2O. The washed beads linked to the unphosphorylated peptide were then combined with antisera and mixed for 6 h at 4°C to adsorb non-Tyr(*P*)-binding polyclonal antibodies with specificity limited to amino acid sequences flanking the critical tyrosine phosphorylation site. After repeated centrifugation to ensure pelleting of the peptide-bound beads, the beads were discarded. Supernatant antisera were then subjected to a second round of RIP to eliminate polyclonal antibodies with specificity limited to Tyr(*P*). These non-receptor-specific antibodies were adsorbed to Affi-Gel 10 chromatography beads (Bio-Rad) linked to phosphotyramine (14). After centrifugation, the supernatant was removed and the purified antibody stored as aliquots at −20°C.

RESULTS

Phosphopeptide Immunogens Yield Antisera Recognizing the Parent Phosphoprotein. Our point of departure was Tyr-1248, which is a major autophosphorylation site of the c-erbB-2 receptor (12, 13). The tyrosine-phosphorylated C-terminal (residues 1243–1255) c-erbB-2 peptide sequence was synthesized. This phosphopeptide was coupled to hemocyanin, gel-purified, and inoculated into rabbits. To identify activation-specific antisera, we needed a cell culture system in which the c-erbB-2 protein could be reversibly converted between the inert and activated configurations. Although initially hindered by the lack of an available receptor-activating ligand, we found that c-erbB-2 receptor activity can be rapidly modified by heterologous transmodulating ligands. When G8/DHFR cells are cultured as density-arrested monolayers, they express a 175-kDa c-erbB-2 protein (p175) that is active as a tyrosine kinase *in vitro* and *in vivo*; within minutes of exposure to platelet-derived growth factor or protein kinase C agonists, however, the activated c-erbB-2 receptor is phosphorylated on serine/threonine residues and converted to a 185-kDa isoform (p185) with markedly reduced tyrosine kinase activity (11).

As shown in Fig. 1*A*, commercially available polyclonal antibodies to the unphosphorylated (residues 1243–1255) peptide recognized both the tyrosine-phosphorylated p175/c-erbB-2 and the transmodulated p185/c-erbB-2. Crude anti-phosphopeptide (apt-1) antiserum preferentially recognized the activated p175 isoform but did not bind as selectively to p175 as did Tyr(*P*) antibody (Fig. 1*A*). Continued immunizations with the phosphorylated peptide increased antiserum

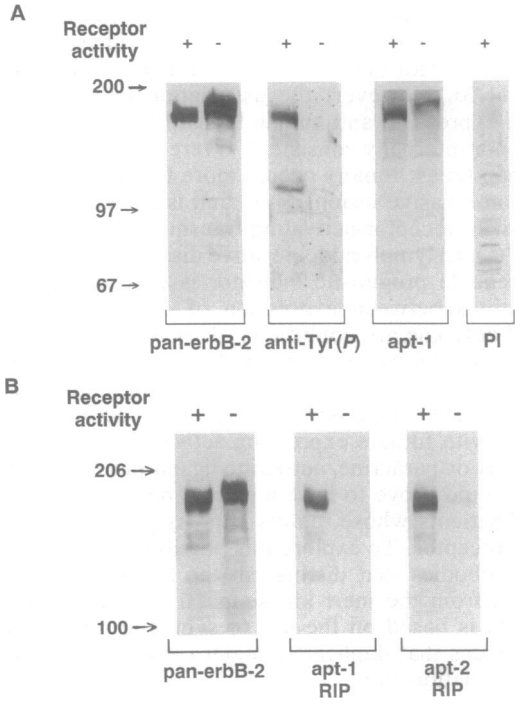

FIG. 1. Isolation of anti-phosphopeptide (apt) antisera. (*A*) Immunoblot analysis of G8/DHFR lysates containing activated (+) and inactivated (−) c-erbB-2 receptors. The pAb-1 rabbit polyclonal antibody, designated here as pan-erbB-2, recognizes both kinase-active and kinase-inactive c-erbB-2 receptor isoforms (11). Preimmune (PI) and crude apt-1 antisera were used at a 1:1000 dilution in TBST. (*B*) RIP of non-activation-specific apt antisera. The unphosphorylated cysteine-linked c-erbB-2 peptide (residues 1243–1255) was bound to Affi-Gel 501 beads. Purified antisera from different rabbits are designated apt-1 and apt-2, with RIP indicating RIP against the unphosphorylated peptide (residues 1243–1255). Molecular masses in kDa are indicated.

immunoreactivity for both anti-p175 and anti-p185, leading to apparent loss of preferential p175 binding; these higher-titer antisera were readily purified, however, by a single-step RIP (data not shown). To establish feasibility of chromatographic purification of the crude anti-p175 antiserum, immunoblot analysis of c-erbB-2-containing lysates was carried out after preincubating antibody solutions with various peptides. These experiments yielded near-monospecificity of apt-1 for p175 (R.J.E., unpublished observations). Similarly, virtual monospecificity of apt-p175 binding was apparent after single-step chromatographic reverse purification of crude apt antisera (Fig. 1*B*).

apt Antiserum Binding to Tyrosine-Phosphorylated Proteins Is Receptor-Specific. Immunization with tyrosine-phosphorylated proteins may yield nonspecific anti-Tyr(*P*) antibodies (16). For this reason, we sought to exclude non-receptor-specific apt antibody binding as a potential confounding factor. Preincubation of apt-1 antisera for 30 min with 1 mM Tyr(*P*) caused no apparent abrogation of apt-1/p175 immunoreactivity (data not shown). Similarly, unpurified apt-1 antiserum failed to recognize tyrosine-phosphorylated EGF receptors or v-src-tyrosine-phosphorylated substrates (Fig. 2*A*). These results indicate that p175 recognition

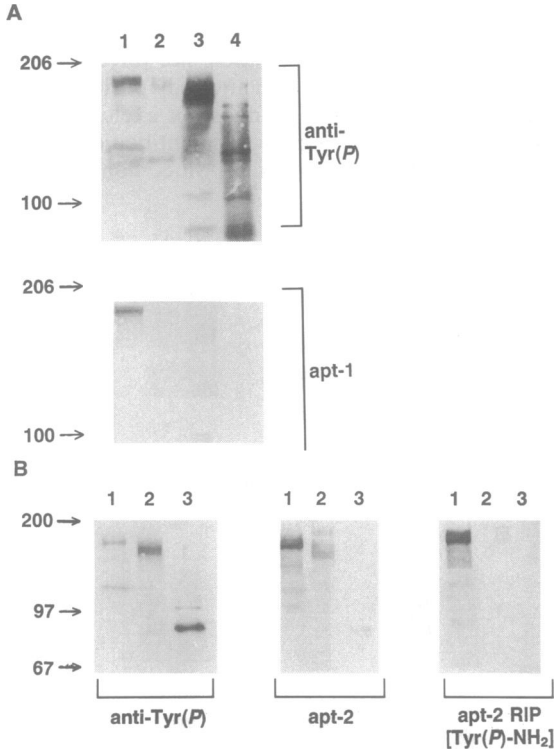

FIG. 2. Exclusion of non-receptor-specific Tyr(*P*) binding. (*A*) Anti-erbB-2 immunoreactivity of apt-1 antiserum after a 6-h incubation with chromatography beads cysteine-linked to the unphosphorylated peptide (residues 1243–1255). Either purified apt-1 antibodies or anti-Tyr(*P*) antibodies were applied to immunoblots of G8/DHFR lysates containing activated (lane 1) or inactivated (lane 2) c-erbB-2 receptors at a 1:500 dilution for 60 min prior to washing, application of alkaline phosphatase-linked second antibody, and colorimetric development. Non-erbB-2-containing Tyr(*P*) controls were provided by EGF-stimulated A431 (lane 3) and v-*src*-transformed cell lysates (lane 4). (*B*) Purification of non-receptor-specific apt-2 antiserum by using RIP and phosphotyramine-linked chromatography (Affi-Gel 10) beads. Phosphotyramine [Tyr(*P*)-NH_2] was prepared and used for reverse chromatographic purification of nonspecific anti-Tyr(*P*) immunoreactivity. In addition to EGF-stimulated A431 cells (lanes 2), the recombinant-activated lck tyrosine kinase (lanes 3) was used as a control. Lanes 1 contain G8/DHFR cells. Molecular masses in kDa are indicated.

by apt-1 antisera does not reflect major contamination with anti-Tyr(*P*) antibodies. Antiserum from a second rabbit (apt-2 antiserum) exhibited binding to Tyr(*P*)-containing EGF receptors and recombinantly produced lck proteins in addition to p175, but this binding was abolished by RIP of apt-2 antisera with Affi-Gel 10 beads linked to phosphotyramine (Fig. 2*B*).

Tyrosine Kinase Activity of c-erbB-2 Correlates with Recognition by apt Antibodies. Tyrosine phosphorylation alone does not necessarily signify increased enzymatic activity of a given tyrosine protein kinase (17). We therefore sought to establish the relationship between apt-1 antibody immunoreactivity and c-erbB-2 receptor tyrosine autophosphorylation activity *in vitro*. For these experiments, c-erbB-2 was immunoprecipitated using a monoclonal antibody recognizing the extracellular domain of the receptor. G8/DHFR cell samples were treated with phorbol ester (which transmodulates the c-erbB-2 receptor; refs. 11 and 18) in the presence or absence of vanadate, lysed, and allowed to autophosphorylate. Samples treated with phorbol ester with and without vanadate exhibited ≈30% and 5% of control receptor kinase activity, respectively (Fig. 3*A*). This corresponds to major reductions in apt-1 binding (Fig. 3*B*). ^{32}P-labeling experiments of phorbol ester-treated G8/DHFR cell cultures confirmed that this reduction in apt-1 binding corresponds to loss of c-erbB-2 tyrosine autophosphorylation activity *in vivo* (Fig. 3*C*).

Heterogeneity of c-erbB-2 Receptor Activation in Human Tumor Cell Lines Is Detected with apt Antibodies. Lysates from five human tumor cell lines (four breast and one ovarian carcinoma line) were probed with a pan-erbB-2 polyclonal antibody (pAb-1). Identical lysates were probed with apt antisera. As shown in Fig. 4, the commercial antisera detected c-erbB-2 protein in all five cell lines. However, only one of the five lines (BT-474) expressed a form of c-erbB-2 readily recognized by apt antibodies.

The basis of this differential reactivity is indicated in Fig. 5. In these experiments, the activation status of c-erbB-2 in the SK-Ov-3 ovarian carcinoma cell line was compared with that of BT-474 cells. In duplicate experiments measuring total c-erbB-2 protein, BT-474, and SK-Ov-3 cells expressed comparable amounts of c-erbB-2 protein. However, only the BT-474 cell line expresses c-erbB-2, which is both highly immunoreactive with anti-Tyr(*P*) (Fig. 5*A*) and active in the *in vitro* kinase assay (Fig. 5*B*). As shown in Figs. 4 and 5*B*, only the BT-474 protein exhibits major immunoreactivity with apt antibodies. Discrimination between these cell lines is also possible by immunoprecipitation with apt-2 and apt-3, but not apt-1, antibodies (data not shown).

DISCUSSION

Our results indicate the feasibility of activation-specific phosphoprotein immunodetection (APHID) by using antibodies raised against synthetic phosphopeptide immunogens. For the c-erbB-2 receptor tyrosine kinase, this capability is particularly attractive given the long-standing absence of an activating ligand for *in vitro* receptor analyses. As a parenthetical observation, we note that many widely used c-erbB-2 antibodies have been raised against the unphosphorylated (residues 1243–1255) c-erbB-2 peptide (2, 4, 19–22) and that these antibodies preferentially—though not exclusively—detect inactivated receptor (R.J.E., unpublished observations). Systematic development of apt antibodies against these and other differentially activated phosphoprotein isoforms using the APHID approach may, therefore, provide insights into this field of tumor biology.

At present we can only speculate as to the significance of differential receptor activity in c-erbB-2-overexpressing human carcinoma cells. Medium-switching experiments do not

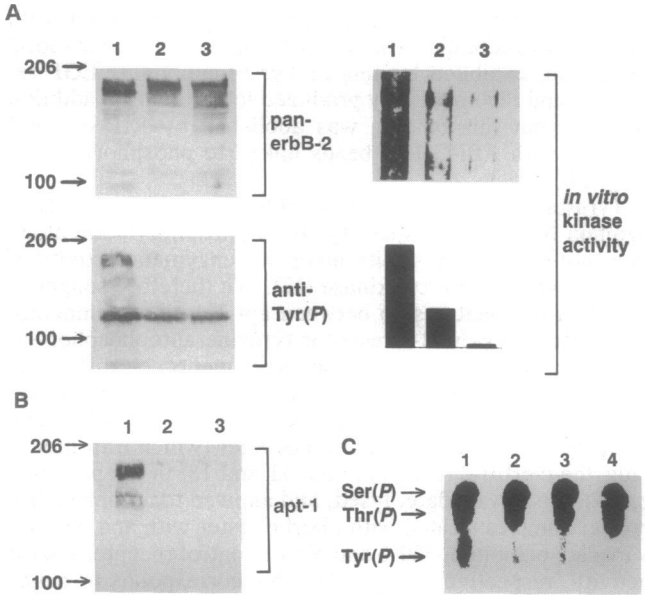

FIG. 3. Confirmation of activation-specific c-erbB-2 receptor binding. (*A*) Characterization of G8/DHFR protein lysates (lanes 1) with respect to c-erbB-2 receptor expression, Tyr(*P*) content, and *in vitro* tyrosine kinase activity. Confluent G8/DHFR cell samples in lanes 2 and 3 were treated with phorbol dibutyrate (100 ng/ml) for 15 min prior to lysis in the presence (lane 2) or absence (lane 3) of the tyrosine phosphatase inhibitor sodium orthovanadate. All manipulations were carried out at 4°C. Cell samples were lysed in LB and immunoprecipitated. Immunoprecipitates were washed twice with LB, twice with distilled H_2O, and twice with KB, then drained, resuspended in 40 μl of kinase buffer containing 0.5 μl of [γ-^{32}P]ATP (specific activity, 3000 Ci/mmol), and incubated at 4°C for 20 min. The reaction was stopped by addition of 1 ml of ice-cold LB. The beads were washed once, resuspended in 50 μl of sample buffer, boiled for 5 min, and loaded onto a 7.5% polyacrylamide gel. After fixation and drying, the gel was exposed and analyzed using PhosphorImager model 400S, and band quantification was carried out using IMAGEQUANT Version 2.0 software (Molecular Dynamics). Bars below lanes on the right measure erbB-2 tyrosine kinase activity in arbitrary units. (*B*) Comparison immunoblot of identical cell lysates using apt-1 antisera. Lanes: 1, G8/DHFR cells; 2, G8/DHFR cells plus phorbol dibutyrate for 15 min; 3, G8/DHFR cells plus phorbol dibutyrate lysed in the absence of vanadate. (*C*) Phosphoamino acid analysis of phorbol ester-treated G8/DHFR cells. *In vivo* phospholabeling was carried out as described (11). Ser(*P*), phosphoserine; Thr(*P*), phosphothreonine. Lanes: 1, control; 2, 10% serum; 3, platelet-derived growth factor; 4, phorbol 12-myristate 13-acetate. Molecular masses in kDa are indicated.

support an autocrine mechanism for BT-474 receptor activity (data not shown), thus leaving open the possibility of a mutation-based activating mechanism in this cell line. Recent studies by other workers (23) have implicated high endogenous tyrosine phosphatase activity as a factor responsible for the relatively low c-erbB-2 receptor activity in SK-Br-3 cells. However, the low receptor activity apparent in SK-Ov-3, MDA-MB-453, and MDA-MB-361 cells cannot be assumed to reflect receptor inactivity in the tumors from which they were derived, since paracrine ligand production may have been operative *in vivo* but absent *in vitro*.

Several groups have isolated antibodies capable of distinguishing tyrosine-phosphorylated (24–26) and serine/threonine-phosphorylated protein isoforms (27). However, these isolations appear to have been at least partly serendipitous, and the procedures employed do not provide a systematic approach to this task. A more methodical strategy has been described by Czernik *et al.* (28) who prepared serine/threonine-phosphorylated immunogens by using protein kinases to phosphorylate synthetic peptides *in vitro*. A limitation of this

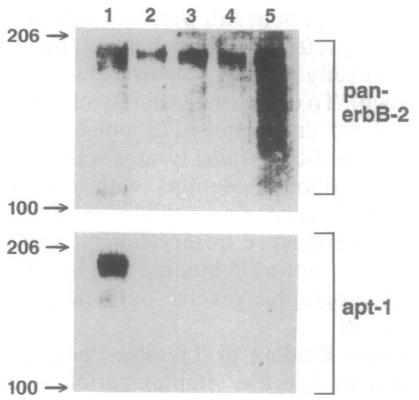

FIG. 4. Identification of heterogeneous apt-1 immunoreactivity in c-erbB-2-overexpressing human tumor cell lines. Binding of pan-erbB-2 (*Upper*) and apt-1 (*Lower*) antibodies in BT-474 (lane 1), SK-Br-3 (lane 2), MDA-MB-453 (lane 3), MDA-MB-361 (lane 4), and SK-Ov-3 (lane 5) cell lines. Cell samples were lysed, electrophoresed, and analyzed on an immunoblot. ^{125}I-labeled anti-rabbit antibody binding was detectable as shown by short (15 min) PhosphorImager exposures; some apt-1 binding was also demonstrable in SK-Br-3 and MDA-MB-453 cell samples on longer exposures (data not shown). Molecular masses in kDa are indicated.

technique is apparent when the desired peptide contains more than one consensus phosphorylation site for the particular kinase. Moreover, the polyclonal antibody purification strategy described by these authors does not exclude the possibility of isolating non-protein-specific antibodies to a single phosphorylated amino acid residue. Since nonspecific anti-

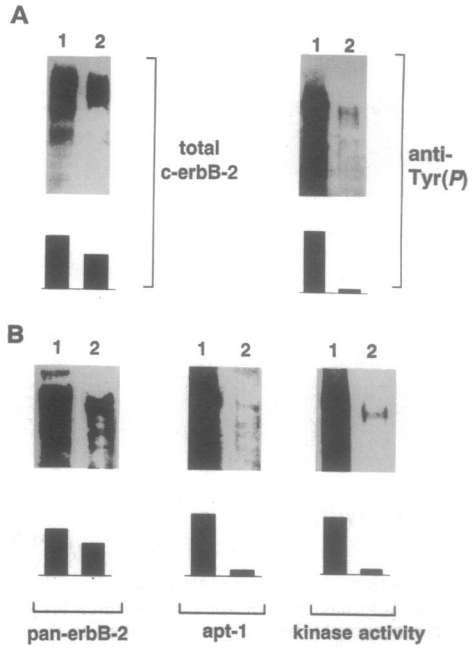

FIG. 5. Quantification of antibody binding and confirmation of heterogeneous c-erbB-2 receptor tyrosine kinase activity in human carcinoma cell lines. (*A*) Quantification of total c-erbB-2 and Tyr(*P*) immunoreactivity in identical BT-474 breast cancer cells (lanes 1) and SK-Ov-3 ovarian cancer cells (lanes 2). Binding of these primary antibodies was quantified by ^{125}I-labeled anti-rabbit and anti-mouse IgG antibody phosphorescence on Phosphor Imager analysis, respectively. (*B*) Detection and quantification of *in vitro* tyrosine kinase activity of BT-474 (lanes 1) and SK-Ov-3 (lanes 2) cells and comparison with total c-erbB-2 expression and apt-1 immunoreactivity. ^{125}I-labeled anti-rabbit antibodies were used for quantification of primary antibody binding. (*A* and *B*) Bars below the lanes show extent of antibody binding (*A* and *B*, first and second gels) and tyrosine kinase activity (*B*, third gel) in arbitrary units.

Tyr(*P*) antibodies are readily detectable in crude antisera of most animals immunized with phosphopeptides to date (Fig. 2 and unpublished data), we believe that the APHID technique provides a highly specific approach to activation-state-specific antibody production.

Can immunization with informative synthetic phosphopeptides, as described here, be extended to other regulatory phosphoproteins? Given that all three rabbits in the present study produced antisera with the desired specificity after purification, we think the prospects are favorable. However, there are certain caveats associated with this approach that emphasize the necessity of corroborating both the activation-specificity and receptor-specificity of other APHID antibodies on a case-by-case basis. (*i*) Immunization with a phosphopeptide could be thwarted by host tyrosine phosphatase activity. Such "off-enzymes" are highly potent inhibitors of c-erbB-2 receptor activation *in vivo* (29) and *in vitro* (23). (*ii*) Phosphorylated amino acid sequences may fail to define immunogenic epitopes distinct from those defined by their unphosphorylated counterparts. This concern reflects published data (30) showing that peptide antibodies raised against unphosphorylated receptor autophosphorylation sequences do not necessarily distinguish such protein isoforms. (*iii*) A third concern relates to the evolutionary conservation of peptide phosphorylation sequences among structurally related protein species: sequence homology has been well-documented for the catalytic domains of >30 tyrosine kinase molecules (31), and a similar degree of homology is apparent within the tyrosine autophosphorylation sites of the c-erbB-2 and EGF receptors (32). Hence, antibodies directed against critical activation-specific peptide sequences of c-erbB-2 might not be receptor-specific. (*iv*) It should be emphasized that tyrosine phosphorylation of a given protein cannot be assumed to correlate with functional kinase activity. This discrepancy has been reported in a number of EGF-stimulated c-erbB-2-expressing cell lines (Rat-1, SK-Br-3, and KB; refs. 17, 33, and 34), though the putative tyrosine-phosphorylated residue(s) do not appear to have been mapped to a specific peptide sequence. For this reason it is mandatory to verify the association of apt antibody immunoreactivity with functional protein activity in any cell system being analyzed.

Notwithstanding these potential pitfalls, the results presented here suggest that APHID technology provides a promising approach to the systematic development of monoclonal and polyclonal antibodies capable of distinguishing a wide variety of functionally divergent regulatory molecules phosphorylated on tyrosine. Such antibodies may also provide nonisotopic probes for mapping and analyzing protein phosphorylation sites. Pending improved techniques for routine synthesis of serine- and threonine-phosphorylated peptides, the APHID approach should facilitate development of antibodies distinguishing protein isoforms phosphorylated on serine and/or threonine residues. Perhaps of greatest interest, however, are the potential diagnostic and therapeutic applications of APHID technology in certain clinical scenarios.

Note Added in Proof. We have recently been made aware of similar results from the laboratory of David F. Stern, Yale University Medical School (personal communication).

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