Isolation of the Intact Molecule and Ectodomain of C-erbB-2 Oncoprotein From SK-BR-3 Cells and Development of Immunoassays on Microplate

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We isolated both the intact molecule (p185) and the ectodomain (p120) of c-erbB-2 oncoprotein from SK-BR-3 breast tumor cells. The p120 was extracted from the cells by 0.05 M phosphate buffer, pH 7.2, whereas the extraction of the p185 required the presence of a detergent, such as 1% Triton X-100 in 0.05 M Tris buffer. Protease inhibitors were also included in the extraction buffer during the isolation of p185 in order to prevent cleavage of p185 to p120 by an unknown protease apparently also present in the extract. In case there was any p120 in the p185 preparation, the p120 could be separated from p185 by chromatography on a Superose 12 column. Using

the p120 and p185 as calibrators, we have established two microplate sandwich immunoassays: one measures both p185 and p120 (total assay) and the other is specific for the p185. Since capturing and detecting antibodies used in the total assay react against the extracellular domain of the c-erbB-2 oncoprotein, they can therefore be used to measure the p120 in serum and p185 in breast tumor tissue cytosol. On the other hand, the p185 specific assay uses the capturing antibody against the cytosolic domain of the oncoprotein and consequently can only measure p185 in breast tumor tissue cytosol. J. Clin. Lab. Anal. 12:298–303, 1998. © 1998 Wiley-Liss, Inc.

Key words: breast carcinoma; p120; p185; prognosis

INTRODUCTION

The c-erbB-2 gene (HER-2/*neu*) is a member of the class of oncogenes associated with tyrosine protein kinase (1). The c-erbB-2 gene was reportedly amplified in 25–30% of human breast and ovarian cancers (2). Slamon et al. (3) demonstrated that c-erbB-2 amplification is an independent predictor of both disease relapse and overall survival when patients test positive. C-erbB-2 was recently found to be a useful marker in identifying patients who are most likely to benefit from high doses of adjuvant chemotherapy (4). There is a direct concordance between c-erbB-2 gene amplification and overexpression of the c-erbB-2 protein (2). The amplification of the human protooncogene c-erbB-2 has been found invariably associated with increased levels of expression of the c-erbB-2 protein in both breast and ovarian tumors (2–5). This phenomenon was noted as well in gastric, endometrial, and nonsmall cell lung adenocarcinomas. As a consequence, both amplification and overexpression of c-erbB-2 have been found associated with poor prognosis and with short survival and recurrence in various carcinomas (6).

The protein encoded by c-erbB-2 is a 185 kDa transmembrane receptor (p185); it is also a glycoprotein having intracellular, transmembrane, and extracellular domains (7–9). The

ectodomain of the c-erbB-2 oncoprotein (p120) could be proteolytically separated from the intact receptor in several breast tumor cell lines and breast tumor tissues (10–14). The ectodomain of the c-erbB-2 oncoprotein was determined in sera from patients with various carcinomas (Wu et al., submitted) associated with specimens containing highly elevated levels of tumor markers (15,16). These oncoproteins were correlated with dominant tumor markers in serial specimens from individual patients. The measurement of p120 has the potential to be both a prognostic marker and a tumor marker. The possibility of replacing the p185 measurement by the determination of the serum concentration of c-erbB-2 protein would make the whole measurement even simpler. Using serum specimens for c-erbB-2 protein measurement eliminates invasive tissue biopsy procedures. It also allows for the repeated collection of specimens at different times. Since c-erbB-2 gene alterations have been detected in mammary tumors as early as in stage I or II (17), this suggests that all of the subsequent events—the overexpression of the *erbB*-

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2 gene, the release of *c-erbB*-2 protein from tumor cells, and the elevation of serum *c-erbB*-2 protein immunoreactivity—could all appear early as well. This in turn implies that the serum *c-erbB*-2 protein immunoreactivity has the potential to become an early tumor marker critically needed for early tumor detection, especially for breast and ovarian cancers.

In order to continue our study of the clinical utilities of serum p120 and p185 in the breast tumor tissue cytosol to compliment estrogen and progesterone receptor assays as a panel of breast tumor risk factors, we have developed two microplate sandwich assays using the p120 and p185 isolated from the SK-BR-3 cell line and commercially available antibodies against various portions of the oncoprotein molecule. Because only p185 and p120 are found in the breast tumor tissue cytosol and patients' sera, respectively, we hope that we can improve the assay's accuracy by using p185 as the calibrator for the cytosol assay and p120 as a calibrator for the serum assay.

MATERIALS AND METHODS

A commercial serum enzyme immunoassay (EIA) kit for the *c-erbB*-2 oncoprotein (which reacts with both p120 and p185) was purchased from Ciba Corning/Triton Diagnostics (Alameda, CA). The monoclonal antibody against the extracellular domain of human *c-erbB*-2 oncoprotein was purchased from Oncogene Science (Cambridge, MA). Monoclonal anti-ectodomain of *c-erbB*-2 oncoprotein antibody was from Zymed (South San Francisco, CA). The rabbit polyclonal antiserum against the ectodomain of human *c-erbB*-2 oncoprotein was from Diagnostic Biosystems (DBS, Fremont, CA). The rabbit antihuman polyclonal antibodies against the cytosolic domain of the oncoprotein and goat anti-rabbit IgG-HRP conjugate were from Dako (Carpinteria, CA). The BCA protein assay reagent (bicinchoninic acid) was from Pierce Chemical Co. (Rockford, IL). Superose 12 HP 10/30 prepac column was purchased from Pharmacia (Piscataway, NJ). The TMB substrate kit and the protease inhibitor, PMSF (phenylmethanesulfonyl), were from Sigma (St Louis, MO). SK-BR-3, a breast adenocarcinoma cell line from human malignant pleural effusion, was purchased from ATCC (Rockville, MD). Both Immuno Immuno-4 strip of Dynatech (Chantilly, VA) and Maxisorp from Nunc (Naperville, IL) were used in the present study to be coated with capturing antibodies for the development of microplate assays.

Listed below are solutions used in all microplate enzyme immunoassays if not specified:

Coating Buffer: 0.1 M carbonate buffer, pH 9.6

Blocking Solution: PBS containing 1% BSA, 0.05% casein and 0.05% Tween 20, pH 7.2

Sample Diluent: PBS containing 1% BSA, pH 7.2

Wash Solution: PBS containing 0.05% Tween 20

Extraction of p185

1. SK-BR-3 cells (1×10^6 to 1×10^8 cells), after growing for 7 days at 37°C, 5% CO₂ (with one medium change after 3–4 days), were removed from the cell culture flask by trypsinization (0.25% Trypsin, 5 mM EDTA in phosphate-buffered saline [PBS]) and then collected by centrifugation at 15,000 rpm for 5 min (all centrifugation was performed on Beckman JA-17).
2. The cell pellet was suspended in 2 mL 0.05 M Tris buffer, pH 7.5 containing 1% Triton X-100, 0.05 M NaCl, 0.01 M EDTA, 0.01 M iodoacetamide, 0.005 M phenylmethyl sulfonyl fluoride (PMSF), 0.01 M sodium azide. The suspension was placed in a rotator rotating for 3 hr at 4°C.
3. Extract containing p185 was collected by centrifugation at 10,000 rpm for 10 min at 4°C. The pellet was extracted one more time following the same procedure described above.
4. Both supernatants from two extractions were combined and concentrated by dialyzing against 100% ammonium sulfate solution.
5. P185 was stored as ammonium sulphate pellet at –70°C or lyophilized after dialysis.

Extraction of p120

To extract p120, the ectodomain of the oncoprotein, the cell pellet of the SK-BR-3 cell preparation (1×10^8 , same cell preparation used for p185 extraction) was suspended in 10 mL 0.05 M phosphate buffer, pH 7.2 at 4°C in the absence of detergent and protease inhibitors. After rotation for 10 min at 4°C the suspension was centrifuged and the pellet was extracted again under the same condition. Both extracts were combined and concentrated by dialyzing against 100% ammonium sulphate at 4°C. Likewise, p120 was also stored either as ammonium sulphate precipitate solution at –70°C or lyophilized.

Superose 12 HR Chromatography

Separation of p185 from p120 was performed on Pharmacia FPLC automated system using Superose 12 HR 10/30 prepac column (bed volume 34 mL). 200 µL of the extract from SK-BR-3 cells was injected directly onto the column after all insoluble substance was removed by centrifugation. The buffer used for column equilibration and elution consisted of 0.05 M phosphate containing 0.15 M NaCl at pH 6.8. The flow rate was maintained at 0.5 mL/min and 0.5 mL per tube of eluate was collected. Eluates were used directly for assay of *c-erbB*-2 oncoproteins using the Triton serum EIA kit without additional treatment.

Triton EIA Kit

The commercial *c-erbB*-2 EIA kit (no longer available) follows a sandwich test format, which uses an antibody-coated

tube for the quantitative measurement of human *c-erbB-2* oncoproteins (p185 and p120) in both tissue cytosol and serum. Both antibodies react with the extracellular domain of *c-erbB-2* oncoprotein. Serum (0.05 mL) is recommended for this kit but heparin, EDTA, or oxalate plasma can also be used. This assay does not react with the epidermal growth factor receptor.

Assay Specific for p185

Preparation of antibody-coated plate

Maxisorp from Nunc was coated with 100 μ L of Dako polyclonal antibodies (5 μ g/mL in 0.1 M carbonate buffer, pH 9.6) against the cytoplasmic domain of *c-erbB-2* oncoprotein (Table 1). After overnight incubation at 4°C the wells were emptied and blocked by the addition of a 200 μ L blocking solution for 6 hr at rt.

Assay procedure

1. Add 100 μ L serum per antibody-coated microwell. Seal the plate and incubate at rt. for 1 hr with gentle agitation.
2. After washing three times add 100 μ L appropriately diluted detecting monoclonal antibody. Incubate for 1 hr at rt.
3. Wash three times and then add 100 μ L of HRP-antimouse IgG antibody (Dako, 1:2500) and incubate for 1 hr at rt.
4. Wash three times followed by the addition of chromogenic substrate TMB solution for color development. The instructions provided by the kit insert were followed.
5. Read the absorbance at 450 nm on a microtiter plate reader (Molecular Devices, Menlo Park, CA) after stopping the color development with 1 N H_2SO_4 .

The unit assigned to our calibrators was based on the calibration against the Triton EIA assay.

Total Assay (p120 and p185)

Preparation of antibody-coated microplate

Maxisorp from Nunc was coated with 100 μ L of monoclonal antibody against the ectodomain of *c-erbB-2* oncoprotein (1 μ g/mL) from Oncogene Science in 0.1M car-

bonate buffer, pH 9.6. The plate was incubated overnight at 4°C and blocked with 200 μ L blocking solution. After being washed three times the antibody coated microplate was dried by lyophilization and stored in a plastic bag at 4°C.

Assay procedure

1. Add 25 μ L sample diluent and 75 μ L serum per well. Incubate for 1 hr at rt.
2. Wash the plate three times with 150 μ L washing solution.
3. Add 100 μ L of the detecting antibody (rabbit antiserum against the extracellular domain of human *c-erbB-2* oncoprotein from DBS at 1:200 dilution).
4. Add 100 μ L anti rabbit IgG HRP-conjugate (1:2,500 diluted) and incubate for 1 hr.
5. After washing three times add 100 μ L TMB solution to each well, incubate for 15 min.
6. Add 1 N H_2SO_4 to each well to stop color development.
7. Read the absorbance at 450 nm.

RESULTS

Preparation of Calibrators

Many tumor cell lines express the *c-erbB-2* oncoprotein but few contain amounts sufficient for calibrator use. For example, only a few hundred units of *c-erbB-2* oncoprotein could be extracted from the LNCaP cells, a prostate cancer cell line. On the other hand, compared to amounts from LNCaP cells, concentrations of the oncoprotein several hundredfold higher were detected in the SK-BR-3 breast tumor cell line. We also discovered that depending on what buffer we were using for extraction, we could isolate either the p120 ectodomain or the intact molecule of the *c-erbB-2* oncoprotein (p185). In fact, *c-erbB-2* immunoreactivity could be found in the cell medium of the SK-BR-3 cell culture without going through any extraction procedure. Unfortunately, the concentration in the cell medium was too low (approximately 30 U/mL); therefore, no attempt was made to isolate the oncoprotein from this source. Based on both the p185 specific assay and the Superose 12 elution profile, only p120 was found in the medium.

Isolation of p120

We found that when the cell pellet was extracted with 0.05 M phosphate buffer, pH 7.2, only the p120 was released into the solution. As shown in Figure 1A extraction of p120 from the cell pellet was almost instantaneous. Prolonging the extraction time might even result in a slight decrease in the amount of p120 extracted. In fact, by the time the cell pellet was suspended in the buffer, all extractable p120 was already released. As shown in Figure 1B only the p120 was extracted from the SK-BR-3 cells with the 0.05 M phosphate buffer in the absence of a detergent. Apparently more p120 could be

TABLE 1. Antibodies Used for Both the Total and p185-specific Assay Established

Assay	Coated antibody	Detecting antibody
Total assay (p185 + p120)	Oncogene Science, Monoclonal against the ectodomain	DBS Polyclonal against the ectodomain (1:100)
p185 Specific assay	Dako Polyclonal anti-cytosolic domain	Zymed/Triton monoclonal against ectodomain (1:1000)

Fig. 1. Extraction of p120 from SK-BR-3 cells and the *c-erbB-2* immunoreactivity elution profile of the extract. (A) SK-BR-3 cell pellet (from approximately 1×10^6 cells) was suspended in 2 mL 0.05 M phosphate buffer, pH 7.2 at 4°C. Aliquots were withdrawn at various time intervals and subjected for measurement by the Triton EIA assay. (B) it is suggested from the *c-erbB-2* immunoreactivity elution profile of the extract following Superose 12 chromatography that the extract contained only p120. The Triton EIA kit was used to measure *c-erbB-2* oncoprotein immunoreactivity.

obtained from the cell pellet with a second extraction (Table 2). We found that the amount of p120 in the combined fractions of first and second extraction was about 90% of the total amount of extractable p120. After second extraction the yield dropped dramatically on the third and fourth attempts. In fact, the fourth extraction, not listed in the table, only produced about 1,500 units, and only 2,100 units of p185 were obtained from the residual cell pellet with buffer containing Triton X-100. Apparently p120 was cleaved from membrane

TABLE 2. Extraction^a of p120^b from SK-BR-3 Cells

Extraction	Buffer volume (mL)	p120 conc. (U/mL)	Total units
1st extraction	20	1,500	30,000
2nd extraction	10	1,300	13,000
3rd extraction	5	750	3,750
Extraction	Protein (mg/mL)	p120 (U/mL)	Total units
1st extraction, 7 mL	6	10,000	70,000
2nd extraction, 3 mL	15	15,000	45,000

^aApproximately 1×10^8 cells were subjected to extractions.

^bTriton kit was used for p120 measurement.

bound p185. Table 2 also lists the yield of p185 in a separate attempt, which used a smaller volume of extraction buffer. We have no explanation as to why the second attempt produced a much higher yield of p185. However, it appears from the current investigation that there is a sufficient amount of p120 extractable from SK-BR-3 cells to meet the need as calibrator and control for the serum immunoassay.

Extraction of p185

We found that the extraction of p185 from SK-BR-3 cells required the presence of the detergent, Triton X-100, in the extraction buffer. Apparently the transmembrane glycoprotein p185 was membrane-bound. In contrast to the p120 extraction, extraction of p185 was time-dependent. As shown in Figure 2A it took at least three hr of suspension for the extraction to reach the maximum yield. Suspending the cells in the extraction buffer beyond five hr at 4°C resulted in a decrease of p185. Although the decline of the p185 concentration was most likely due to the result of instability of p185 under such diluted protein concentrations, it might also be due to a conversion of p185 to p120. Apparently the protease that is responsible for the cleavage of p185 to p120 was also present in the extract. This is the reason why we routinely included protease inhibitors, such as PMSF, in the extraction

Fig. 2. Extraction of p185 from SK-BR-3 cells and the elution profile of the extract. (A) cell pellet of SK-BR-3 cells (1×10^6) was suspended in 2 mL Tris buffer containing both Triton X-100 and protease inhibitors. It appears that the release of p185 from the cells is time dependent and will take at least 3 hr at 4°C to extract the maximal amount. (B) it is suggested from the *c-erbB-2* immunoreactivity elution profile of the extract following Superose column chromatography that the majority of the *c-erbB-2* immunoreactivity of the extract was derived from p185.

buffer. As shown in the elution profile in Figure 2B, when protease inhibitors were included in the extraction buffer, the majority of the *c-erbB-2* immunoreactivity we detected in the extract was p185. There were some minor amounts of p120 which could easily be eliminated by Superose chromatography.

Assay Specific for p185

With p185 and p120 from SK-BR-3 cells available for use as calibrators, two sandwich-type immunoassays were established in-house. One assay specifically measured the p185 that coated the microplate with polyclonal antibody against the cytosolic domain of the *c-erbB-2* oncoprotein (Table 1). As a consequence, during the sample incubation, only the intact transmembrane glycoprotein would be captured by the coated antibodies; therefore, only the p185 would be detected. The ectodomain p120, lacking the cytosolic domain of the original molecule, would not be detected by this assay. The p185 specific assay used a Zymed/Triton monoclonal antiectodomain of the *c-erbB-2* oncoprotein for detection and an HPR-conjugated antimouse IgG for color development. The use of a pair of poly- and monoclonal antibodies reduced the possibility of a high background and also resulted in a much more sensitive assay. This assay should be useful as a quantitative measurement of p185 in the breast tumor tissue cytosol, and together with ER and PgR, for risk assessment and for helping to select a proper treatment. The calibration curve for the p185 specific assay is shown in Figure 3A. The linear portion of the curve only extended to 75 U/mL. Assaying specimens containing p185 above 100 U/mL will be impacted by the hook effect. This effect can be reduced by either lowering the amount of specimen used or by increasing the amount of antibody coated. The sensitivity of the test was determined to be 3.2 U/mL using a calibrator containing a zero concentration of p185 (3 SD from the mean). The intra-assay precision data are listed in Table 4.

Total Assay Measuring Both p120 and p185

By using a pair of antibodies against two different sites on the ectodomain of the *c-erbB-2* oncoprotein, a sandwich-type immunoassay was developed on microplate which could detect both the ectodomain (p120) and the intact molecule of *c-erbB-2* oncoprotein (p185). The calibration curve is shown in Figure 4. The assay is sufficiently sensitive to measure either the p120 in serum or the p185 in breast tumor cytosol. When used to measure p185 in the breast tumor cytosol p185

TABLE 3. Extraction of p185 From SK-BR-3 Cells

Extraction	Protein (mg/mL)	p185 (U/mL)	Total units	U/mg
1st 1.1 mL	1.2	9400	10,340	7800
2nd 2.5 mL	1.7	6800	17,000	4000

A P 185 Calibration Curves

Fig. 3. Dose response curves of the p185 specific assay. (A) a typical calibration curve using p185 isolated from SK-BR-3 cells and with value assigned by the Triton EIA assay. (B) demonstration that there is hook effect under the current assay conditions.

should be used to construct the calibration curve. The commercial Triton assay used the test tube for antibody coating whereas we used the microplate as the solid phase to coat the antibody. Using a microplate as the solid phase not only saves reagents but also allows the entire assay to be semiautomated. The sensitivity of the total assay was determined to be 7.4 U/mL using a calibrator containing zero concentration of p120 (3 SD from the mean). The intra-assay precision data are listed in Table 5.

DISCUSSION

Although many tumor cell lines express the *c-erbB-2* oncoprotein, it was not until we measured the *c-erbB-2* oncoprotein in the SK-BR-3 cell line that we felt that we had found the source to produce a sufficient amount of oncoprotein to meet the need as calibrator for assay establishment. We also discovered that the amount of oncoprotein present in the cell medium was too low for any practical use. However, it was interesting to learn that based on the elution profile of

TABLE 4. Intra-Assay Precision for p185-Specific Assay

Number of repeats	Mean (U/mL)	SD	CV (%)
24	23.9	1.32	5.5
24	15.2	1.1	7.2

Fig. 4. A typical calibration curve for the total assay (p185 & p120). p120 isolated from the SK-BR-3 cells with value assigned based on the Triton EIA assay was used to prepare the calibration curve.

chromatography on Superose 12 column, the immunoreactivity detected in the cell medium was derived entirely from p120. During our attempt to remove the residual p120 from the cell pellet by washing the cell pellet with the phosphate buffer, we found that a relatively large amount of *c-erbB-2* oncoprotein immunoreactivity could be released into the washing buffer; the immunoreactivity was much higher than what we detected in the cell medium. Again, based on the elution profile of Superose 12 chromatography it was confirmed that all the released immunoreactivity was derived from p120. The release of p120 only by phosphate buffer and in the absence of detergent provided a practical, convenient means for p120 isolation for assay establishment. However, how such a large amount of p120 could be released by phosphate buffer in such a short time remains unclear. Enzymatic cleavage of the transmembrane p185 molecule is supposed to be time-dependent. It is difficult to understand how p120 could be cleaved and released so quickly at 4°C.

On the other hand, extraction of p185 requires the presence of detergent, such as Triton X-100. However, we observed that p185 in the extract was gradually turned into p120. Apparently proteases, responsible for the cleavage of p185 to p120, were also present in the extract. We were finally able to stabilize the p185 preparation in the extract by including protease inhibitors in the extraction buffer. This p185 preparation was suitable for use as a calibrator for the p185-specific assay. It can also be used as both calibrator and control for the total assay if the assay is intended to be used to measure p185 in breast tumor tissue cytosols.

Because only a few hundred units of *c-erbB-2* oncoproteins

are needed for each calibrator, the amount of p185 and p120 isolated from 1×10^8 cells should be sufficient to run thousands of calibration curves. It should be mentioned that there is no need to further purify either p120 or p185 if it is only used as calibrator or control, although one must make certain that the p185 preparation does not contain any p120 or vice versa. As we have demonstrated, p120 and p185 can be conveniently separated by chromatography on a column containing Superose 12.

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TABLE 5. Intra-Assay Precision for Total Assay

Number of repeats	Mean (U/mL)	SD	CV (%)
20	25	1.25	5
20	50	2.25	4.5