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A 67 kDa NON–HORMONE BINDING ESTRADIOL RECEPTOR IS PRESENT IN HUMAN MAMMARY CANCERS

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The presence of large amounts of a 67 kDa estradiol receptor that does not bind hormone was observed in 8 of 37 human mammary tumors (34 malignant and 3 benign). This form of receptor was detected by its conversion to hormone binding receptor by an endogenous tyrosine kinase *in vitro*. All 8 tumors were malignant. In these, the incubation of cytosol with ATP was seen to cause a 1- to 5-fold increase in estradiol-specific binding sites. These sites bound estradiol with physiological affinity, and their appearance was associated with tyrosine phosphorylation of estradiol receptor. The enzyme converting the non–hormone binding receptor into the hormone binding receptor is largely present in cytosol and scarce in membranes. It has been extensively purified. It is a 67 kDa protein under denaturating conditions, binds calmodulin-Sepharose in a Ca^{2+}-dependent manner, is stimulated by Ca^{2+} and calmodulin, phosphorylates exogenous actin, is activated by the estradiol–receptor complex. The enzyme interacts with antibodies directed against the carboxy-terminal and catalytic domains of c-*src*. Therefore, it is a putative new member of the large c-*src*-related kinase family. Human mammary cancers with significant amounts of 67 kDa non–hormone binding receptor show relatively low levels of hormone binding estradiol receptor. The presence of non–hormone binding receptor that can be activated by *in vitro* tyrosine phosphorylation suggests that functional interaction of estradiol receptor with tyrosine kinases is altered in malignant tumors and has bearing on loss of hormone dependence and progression of the mammary cancer malignancy.

Tyrosine phosphorylation of proteins is a relatively rare event involved in cell multiplication induced by growth factors and oncogenic transformation (Bishop, 1985). Many oncogenes code for tyrosine kinase (PTK_s), and their altered expression causes altered tyrosine phosphorylation that is responsible for cell transformation. High levels of cytosol PTK activity in human mammary cancers are associated with shorter disease-free intervals and low levels of hormone binding estradiol receptor (Romain *et al.,* 1994).

Estradiol receptor is phosphorylated *in vivo* at a tyrosine residue in different tissues and under different conditions, as initially observed by our group and subsequently confirmed elsewhere (Migliaccio *et al.,* 1986; Arnold *et al.,* 1995; Auricchio *et al.,* 1995; Pietras *et al.,* 1995). There is strong evidence that cytosol tyrosine phosphorylation and nuclear phosphotyrosine dephosphorylation of ER modulates its hormone binding activity (Auricchio *et al.,* 1987). ER tyrosine phosphorylation at position 537 by a cytosol PTK is crucial for ER hormone binding (Castoria *et al.,* 1993). Most of the *in vitro* synthesized point mutated (HEO) or wild-type (HEGO) human ER is unable to bind hormone unless it is tyrosine phosphorylated by this cytosol kinase (Migliaccio *et al.,* 1989, 1991). In contrast, incubation of ER from normal tissues with the same kinase does not significantly affect hormone binding (Migliaccio *et al.,* 1984; Auricchio *et al.,* 1987). This suggests that in normal tissues most of the ER is present in a phosphorylated, hormone binding form. The presence of NHBER in hormone-independent mammary cancers experimentally induced in mice of the inbred GR strain has been observed (Migliaccio *et al.,* 1992). We now report NHBER in human mammary cancers. This form of receptor characterizes a group of tumors with scarce hormone binding ER. Low levels of ER, mostly assayed by hormone binding alone or in association with immunocytochemistry and mRNA levels, predict reduction of the disease-free interval (Knight *et al.,* 1977; Beck *et al.,* 1994; Nagai *et al.,* 1994).

MATERIAL AND METHODS

Reagents

[^{3}H]estradiol (99 Ci/mmol) was from Amersham (Aylesbury, UK). Rabbit, rat and goat anti-mouse antibodies, protein G–Sepharose, calmodulin and calmidazolium (R24571) were from Sigma (St. Louis, MO). Cellulose nitrate (0.45 μm) was from Schleicher and Schuell (Dassel, Germany). Anti-ER monoclonal antibodies (H222) were a generous gift from Abbott (Abbott Park, IL). Monoclonal anti-phosphotyrosine antibodies, IgG_{2bk} (clone 4 G10), were from UBI (Lake Placid, NY). The anti-RRDP and anti-cst.1 antibodies were a generous gift from Dr. S.A. Courtneidge (Heidelberg, Germany). The monoclonal anti-c-*src* antibody (327 MAb) was kindly provided by Dr. J.S. Brugge (ARIAD Pharmaceuticals, Cambridge, MA). Polyclonal rabbit anti-c-*erbB-2* antibodies (M6) were a generous gift from Dr. P.P. Di Fiore (NCI, Bethesda, MD). Anti-mouse, anti-rabbit and anti-rat IgG, AP-conjugated, were from Promega (Madison, WI). All the other reagents were of analytical grade.

Cytosol and particulate fraction preparation

Human mammary tumor tissue was taken from 36 patients undergoing surgical resection. A relapsed tumor from 1 patient was also analyzed. Soon after removal, the tissue was transported on ice to the laboratory, where it was frozen in

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Abbreviations: ATP, adenosine triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-*bis*(β amino-ethyl ether) *N,N,N',N'*-tetraacetic acid; TCA, trichloroacetic acid; LAP, cocktail of protease inhibitors: leupeptin, antipain, pepstatin, 10 μg/ml of each inhibitor; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; Ab, antibody; MAb, monoclonal antibody; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitroblue tetrazolium; TGD buffer, 50 mM Tris-HCl, containing 1 mM DTT, 0.2 mM EGTA, pH 7.4; TGD-sucrose buffer, TGD buffer containing 0.25 M sucrose; lysis buffer, 50 mM Tris-HCl, pH 7.4, containing 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 1% aprotinin and LAP cocktail; TBST buffer, 10 mM Tris-HCl, pH 8, containing 150 mM NaCl and 0.05% Tween 20 (v/v); blocking solution, TBST buffer containing 3% bovine serum albumin; AP buffer, 0.1 M Tris-HCl, pH 9.5, containing 0.1 M NaCl and 5 mM $MgCl_2$; AP, alkaline phosphatase; DCC, dextran coated charcoal; DMP, dimethyl pimelimidate; PTK, protein tyrosine kinase; PTP, phosphotyrosine phosphatase; ER, estradiol receptor; NHBER, non–hormone binding ER.

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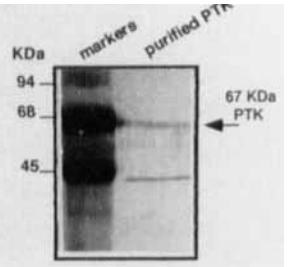
TABLE I – PURIFICATION OF ESTRADIOL-DEPENDENT PTK FROM HUMAN MAMMARY CARCINOMA

Steps [a]	Volume (ml)	Total protein (mg)	Total activity (arbitrary units) [b]	Purification factor	Yield (%)
Heparin-Sepharose (flow-through)	50	12.6	51.8	1	100
DEAE-cellulose	6	0.96	25.9	6.5	50
Calmodulin-Sepharose	3	< 0.005 [c]	19.1	> 5660 [c]	36



[a]The initial 2 steps of purification, cytosol preparation and ammonium sulphate fractionation, are not reported in the table because activity was not assayed at these stages.–[b]PTK activity is expressed as arbitrary units. One arbitrary unit is the activity activating 10 fmoles of estradiol-specific binding sites/min–[c]Protein concentration is below the lowest limit of the assay (1 μg/0.8 ml). Maximal protein concentration and minimal purification factor of the PTK eluted from calmodulin-Sepharose are calculated on this basis.–Insert panel: Silver nitrate staining of the PTK eluted from calmodulin-Sepharose and resolved on an 11% SDS-PAGE gel.

TABLE II - EFFECT OF ATP ON SPECIFIC [^{3}H]ESTRADIOL BINDING IN CYTOSOL OF HUMAN MAMMARY TUMORS[1]

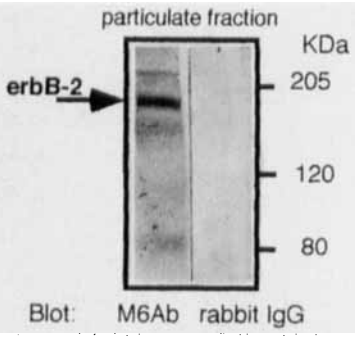
No.	Specific [^{3}H]estradiol binding sites		ATP-activated binding sites		Histology	Age (years)
	−ATP	+ATP	fmoles/mg protein	Increase over the basal level (%)		
1	32	46	14	44	Infiltrating ductural carcinoma	67
2	**45**	**151**	**106**	**235**	**Infiltrating ductural carcinoma**	**72**
3	57	47	−10	−13	Infiltrating ductural carcinoma	40
4	**38**	**245**	**207**	**545**	**Infiltrating ductural carcinoma**	**70**
5	29	29	0	0	Lobular carcinoma	62
6	**15**	**95**	**80**	**523**	**Infiltrating ductural carcinoma**	**66**
7	100	147	47	47	Infiltrating ductural carcinoma	49
8	134	183	49	37	Infiltrating ductural carcinoma	58
9	**16**	**91**	**75**	**468**	**Infiltrating ductural carcinoma**	**74**
10	**74**	**165**	**91**	**123**	**Infiltrating ductural carcinoma**	**61**
11	149	154	5	4	Infiltrating ductural carcinoma	66
12	**24**	**89**	**65**	**270**	**Infiltrating ductural carcinoma**	**57**
13	5	0	−5	—	Infiltrating ductural carcinoma	47
14	30	17	−13	−44	Infiltrating ductural carcinoma	43
15	26	39	13	50	Infiltrating ductural carcinoma	42
16	50	52	2	4	Infiltrating ductural carcinoma	63
17	153	130	−23	−16	Infiltrating ductural carcinoma	54
18	36	48	12	33	Infiltrating ductural carcinoma	30
19	170	179	9	5	Carcinomatous mastitis	34
20	363	412	49	13	Infiltrating ductural carcinoma	61
21	22	14	−8	−37	Fibrous dysplasia	57
22	68	44	−14	−36	Infiltrating ductural carcinoma	54
23	43	50	7	16	Infiltrating ductural carcinoma	54
24	60	72	12	20	Carcinomatous mastitis	53
25	792	754	−38	−5	Infiltrating ductural carcinoma	66
26	11	7	−4	−37	Intraductural papilloma	43
27	74	67	−7	−9	Infiltrating ductural carcinoma	42
28	42	57	15	35	Infiltrating ductural carcinoma	63
29	541	514	−27	−5	Infiltrating ductural carcinoma	54
30	428	458	30	7	Infiltrating ductural carcinoma	78
31	440	496	56	12	Fibroadenoma	45
32	**47**	**149**	**102**	**217**	**Infiltrating ductural carcinoma**	**48**
33	9	40	31	365	Infiltrating ductural carcinoma	41
34	123	153	30	24	Infiltrating ductural carcinoma	51
35	85	117	32	38	Infiltrating ductural carcinoma	61
36	99	135	36	36	Infiltrating ductural carcinoma	31

[1]Binding is expressed as fmoles/mg protein. In bold type are tumors with a large increase of ATP-activated binding sites in terms of both fmoles/mg of protein and increase over the basal level (see Results). The assays of ATP-activated binding sites shown in the table were performed in duplicate. No. represents the tumor identification number. It is used throughout the paper.

liquid nitrogen for no longer than 2 weeks. Bovine uteri from young calves were submitted to the same treatment as tumor tissues. Homogenization of bovine uteri and mammary tumors in TGD-sucrose buffer and cytosol preparation were performed as previously reported (Migliaccio *et al.*, 1992; Castoria *et al.*, 1993). The nuclear supernatant was centrifuged at 105,000 *g* for 45 min at 2°C; the pellet was employed as the particulate fraction and the supernatant of the particulate fraction was used as the soluble fraction. The particles were washed with 10 ml of TGD-sucrose buffer and suspended in 0.5 ml of lysis buffer. Both fractions were used either for Western blot (see the section on Immunoblots with anti-c*src* or anti-c-*erbB-2* antibodies) or for PTK-activity assay (see the Kinase purification and PTK-activity assay section). When the particulate fraction was assayed for enzymic activity, it was suspended in 0.5 ml of lysis buffer modified as follows: Triton

TABLE III – NHBER IN HUMAN MAMMARY CANCER BEFORE AND AFTER RELAPSE

Tumor number	Specific [^{3}H]-estradiol binding sites[1]		ATP-activated binding sites[1]
	−ATP	+ATP	
3	57	47	- 10
3 **	46	95	49



[1]Expressed as fmoles/mg cytosol protein.–**Relapsed after 1 year.–Insert panel: The pellet at 105,000 *g* was reduced in Laemmli sample buffer, then resolved by SDS-PAGE and blotted with anti-c-*erB-2* Ab (M6 Ab). The position of c-*erB-2* is indicated by the arrow.

X-100 concentration was decreased to 0.1% and EDTA substituted by 0.2 mM EGTA.

Assay of ATP-activated binding sites of ER

The assay to determine ATP activation of ER from mammary tumor cytosols, the study on estradiol dependence of ATP-activated binding sites and the measurements of estradiol-specific binding sites by DCC treatment were performed as reported (Migliaccio *et al.*, 1992).

Kinase purification and PTK-activity assay

The calf uterus PTK was isolated as previously reported (Castoria *et al.*, 1993). The PTK from human mammary tumors was purified according to the same procedure, with minor modifications. The 2 final steps of the original procedure, poly-(Glu-Tyr)-Sepharose and ATP-agarose chromatographies, which only slightly improved the final purification, were omitted. Seven grams of tumor tissue was homogenized in 10 volumes instead of 4 volumes of TGD-sucrose buffer, and the DEAE-cellulose column (2.5 ml) was step-eluted by 6 ml of TGD buffer containing 175 mM KCl, pH 7.4, in place of a linear salt gradient elution. A Bio-Rad (Richmond, CA) protein assay kit was used. The purified PTK was stored in 40% glycerol at −20°C for no longer than 3 days. The PTK-activity assay and its estradiol dependence were monitored as previously reported using nuclear PTP-inactivated bovine ER as a substrate and measuring the ATP-activated binding sites of this ER (Auricchio *et al.*, 1987; Castoria *et al.*, 1993). No activation of hormone binding sites by the purified enzyme was detected in the absence of estradiol (not shown). Table I summarizes the purification procedure for PTK from human mammary cancer. The crucial step is represented by the calmodulin-Sepharose chromatography. The enzyme interaction with calmodulin is Ca^{2+}-dependent, as indicated by its occurrence in the presence of Ca^{2+} and elution of the PTK from the column by an excess of EGTA. The insert gel panel in Table I shows silver nitrate staining of the TCA-precipitated purified PTK submitted to 11% SDS-PAGE. Two main proteins were detected, migrating at 67 and 43 kDa, respectively. From a previous study on uterus PTK, we know that the kinase is a 67 kDa protein (Castoria *et al.*, 1993).

Actin tyrosine phosphorylation assay

This assay was performed as previously reported through the step of incubating either kinase, ER or ER and kinase with actin (Castoria *et al.*, 1993). After incubation, the proteins were precipitated by TCA, reduced by heating in Laemmli sample buffer and resolved (40 µl) on an 11% SDS-PAGE gel. Proteins were transferred to a nitrocellulose filter as reported (Towbin *et al.*, 1979) and immunoblotted with anti-phosphotyrosine antibody (Auricchio *et al.*, 1995). Pure ER used in this experiment was prepared as previously reported (Abbondanza *et al.*, 1993). In the final step, the preparation was applied to AER 311 MAb–protein G–agarose, DMP-linked and eluted hormone-free at pH 12. Pure ER was then equilibrated at 2°C for 2 hr with 10 nM of cold estradiol and used. AER 311 MAb was raised against calf uterus ER.

*Assay of PTK activity precipitated by anti-c-*src *Ab*

Aliquots of cytosol (1 mg protein/ml) from bovine uterus and human mammary cancers were incubated either with 5 µg of rabbit or with 2 µg of goat anti-mouse Ab for 1 hr at 2°C in the presence of the protease inhibitors in the LAP cocktail. Then 50 µl of protein G–Sepharose suspension (50% in TGD buffer) was added to each sample, and incubation continued for an additional hour at 2°C. After centrifugation, the supernatants (900 µl) were collected and incubated with anti c-src Ab (either 10 µg of rabbit anti-cst.1 Ab or 10 µl of mouse 327 MAb). Control samples containing either 10 µg of rabbit or 2 µg of goat anti-mouse Ab in place of anti-cst.1 Ab or 327 MAb, respectively, were run in parallel. After shaking at 2°C for 2 hr, 1 µg of goat anti-mouse Ab was added to the samples incubated with 327 MAb. Then, 100 µl of protein G–Sepharose suspension (50% in TGD buffer) was added to each sample. After 30 min at 2°C, pellets were collected by centrifugation and washed 2 times with 1 ml of TGD buffer containing 0.4 M KCl and 2 times with TGD buffer. The final pellets were gently suspended in PTK assay buffer (500 µl of TGD buffer containing 10 µg of LAP protease inhibitors, 20 mM Na_2MoO_4, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 20 µg/ml calmodulin), and PTK activity precipitated by anti-c-*src* or control Ab was assayed as ATP-dependent estradiol-specific binding site activation using PTP-inactivated ER as previously reported (Castoria *et al.*, 1993). The PTK activity specifically associated with anti-c-*src* Ab immunoprecipitates was calculated as the difference between PTK activity associated with anti-c-*src* Ab and that with control Ab.

*Immunoblots with anti-c-*src *or anti-c-*erbB-2 *antibodies*

PTK was isolated from 150 g of bovine uterus as previously reported (Castoria *et al.*, 1993) or purified from human mammary carcinoma as reported in the Kinase purification and PTK-activity assay section. The preparations were reduced by heating in 100 µl of Laemmli sample buffer, and 25 µl of each sample was resolved on an 11% SDS-PAGE gel using a minigel apparatus (Bio-Rad). Electrophoretically separated proteins were transferred to nitrocellulose and blotted with either anti-c-src Ab (anti-cst.1, anti-RRDP, 327 MAb) or

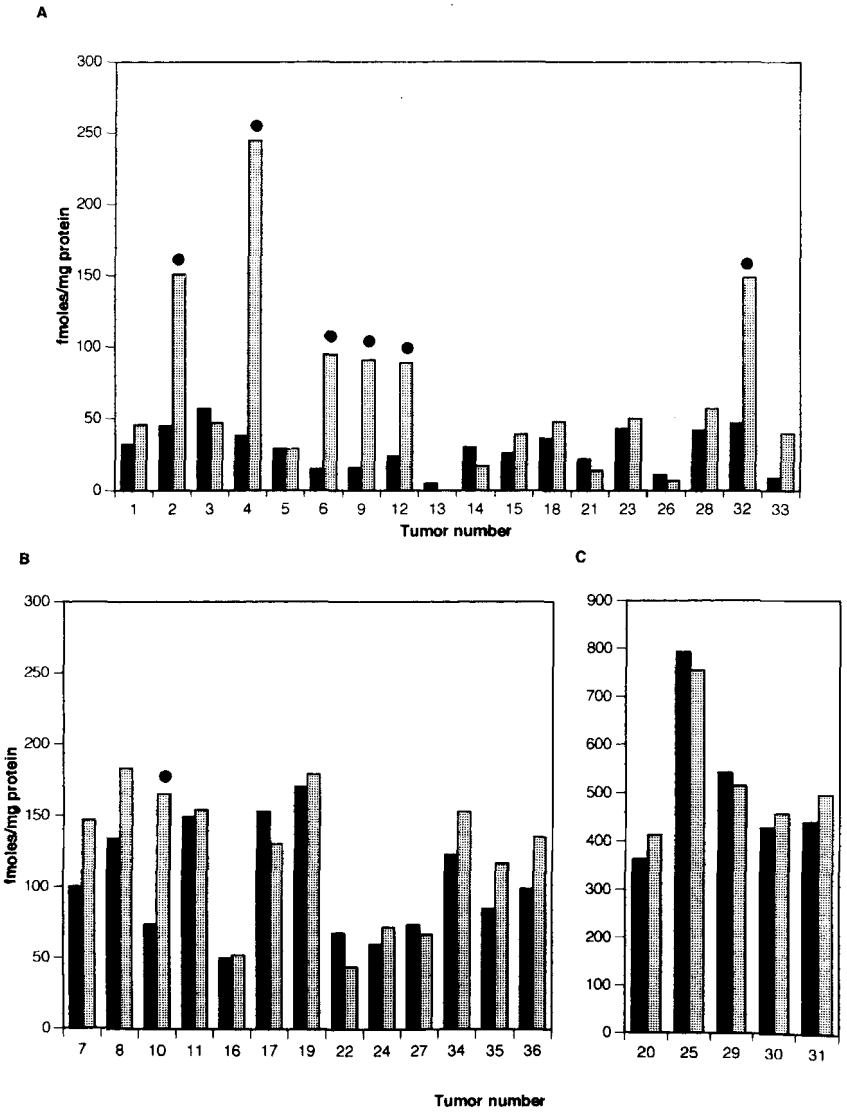


FIGURE 1 – ATP-dependent activation of specific estradiol binding sites in relation to the cytosol basal binding sites. Data are those listed in Table II. Tumor cytosols with estradiol binding/mg of protein of *(a)* less than 50 fmoles/mg of protein, *(b)* from 50 to 300 fmoles/mg of protein and *(c)* more than 300 fmoles/mg of protein. Dark bars, tumor cytosols incubated without ATP; shaded bars, tumor cytosols incubated with ATP. The symbol ● at the top of the bars indicates tumors with a large increase of ATP-activated binding sites.

rabbit Ab (dilution of 1:1000 in blocking solution for anti-cst.1, anti-RRDP and rabbit Ab; dilution of 1:50 in TBST for 327 MAb) for 2 hr. Filters were washed 4 times in TBST buffer and then incubated with anti-rabbit or anti-mouse Ab AP-conjugated for 30 min (dilution of 1:7500 in blocking solution and 1:7500 in TBST buffer for anti-rabbit or anti-mouse Ab, respectively). Filters were then washed 4 times with TBST buffer, and the phosphatase activity was revealed using BCIP and NBT. Soluble and particulate fractions from human mammary tumors were prepared as described in a previous section. Aliquots of 100 µl from both (each containing 110–120 µg of protein) were reduced in Laemmli sample buffer and resolved (25 µl) on an 11% SDS-PAGE gel using a minigel apparatus (Bio-Rad). Electrophoretically separated proteins were immunoblotted with either rabbit, or anti-RRDP antibodies. When indicated, aliquots of 100 µl from the particulate fraction were reduced in Laemmli sample buffer and resolved (60 µl) in duplicate on an 8% SDS-PAGE gel. Electrophoretically separated proteins were then transferred to nitrocellulose and finally probed with either rabbit or anti-c-*erbB-2* Ab (M6) (Auricchio *et al.*, 1994).

In vitro tyrosine phosphorylation of ER by the cytosol PTK

Two cytosol aliquots from human mammary cancer (each of 1 ml containing 2 mg of protein) incubated in the absence or presence of 0.15 mM ATP (Migliaccio *et al.*, 1992) were treated with 50 µl of protein G–Sepharose suspension (50% in TGD buffer). Supernatants were collected by centrifugation and incubated with anti-ER (H222) MAb or rat IgG (control), as reported previously (Auricchio *et al.*, 1995). The immunoprecipitates were reduced with Laemmli sample buffer and resolved (30 µl) on 11% SDS-PAGE gels. Proteins electrophoretically separated were transferred to nitrocellulose membranes and blotted with either anti-ER (H222) MAb or anti-phosphotyrosine MAb, according to the same report. The estradiol dependence of tyrosine phosphorylation of the 67 kDa ER was analyzed by incubation of cytosol samples with 0.15 mM ATP, either in the presence or in the absence of 10

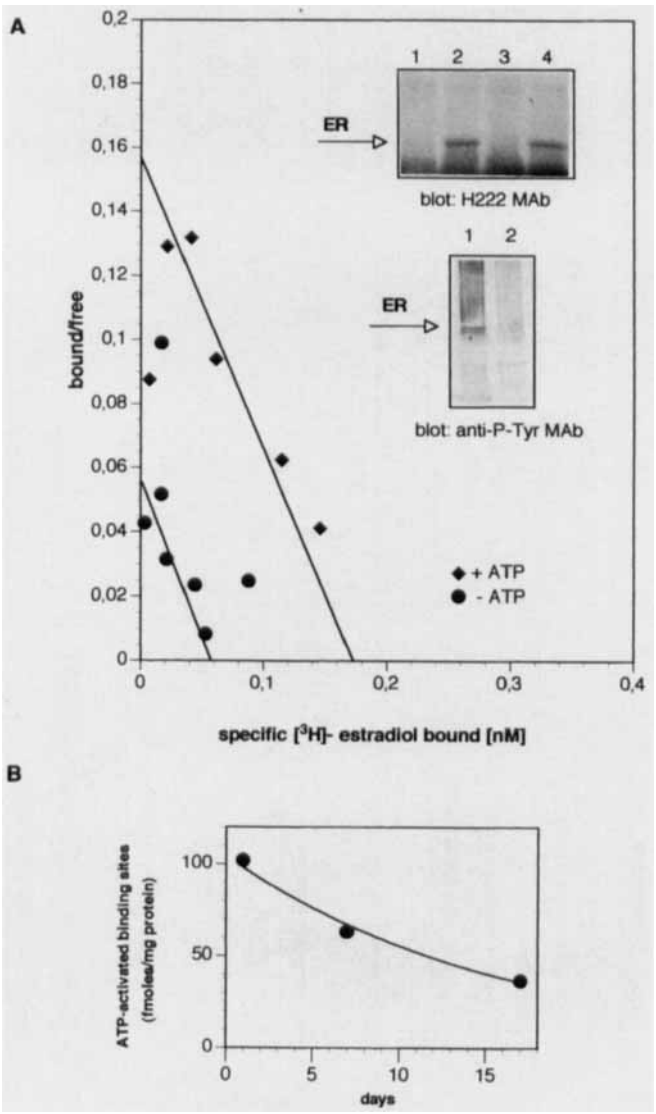


FIGURE 2 – Scatchard analysis of estradiol binding sites before and after ATP-dependent activation and tyrosine phosphorylation of the cytosol ER from human mammary cancer *(a);* stability of the enzyme responsible for the ATP-dependent activation of binding sites *(b). (a)* Aliquots of cytosol (400 µl) were equilibrated for 2 hr, in the presence or absence of an excess of cold estradiol, with different concentrations of [^{3}H]estradiol. ATP-activated binding sites were measured as described in the Material and Methods section. Upper insert: Blot with H222 MAb of human mammary cancer cytosol incubated without (lanes 1 and 2) or with (lanes 3 and 4) ATP and immunoprecipitated with control Ab (lanes 1 and 3) or H222 MAb (lanes 2 and 4). Lower insert: Blot with anti-phosphotyrosine MAb of human mammary cancer cytosol incubated with (lane 1) or without (lane 2) ATP and immunoprecipitated with H222 MAb. Prestained molecular weight markers were run and transferred in parallel (not shown). The arrows indicate the position of the 67 kDa ER. *(b)* Tissue from the same cancer used in the experiment presented in *(a)* was frozen in liquid nitrogen for 17 days. At the indicated intervals, a sample was removed and thawed; cytosol was prepared and ATP-activated binding sites were monitored.

nM cold estradiol, as described (Migliaccio *et al.,* 1992). The H222 MAb immunoprecipitation of the resulting samples as well as blotting of the immunoprecipitates with anti-phosphotyrosine MAb were performed as reported (Auricchio *et al.,* 1995).

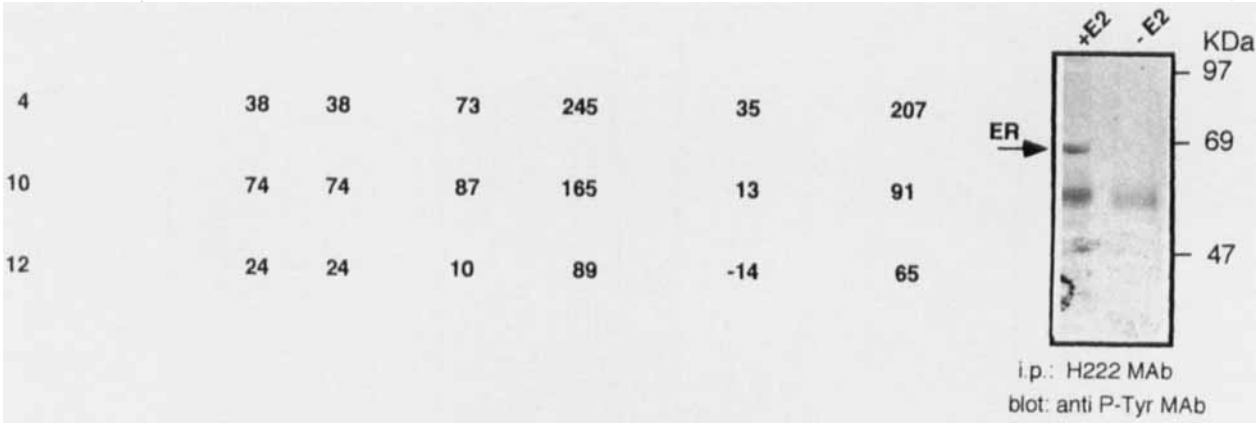
RESULTS

Effect of ATP on [^{3}H]estradiol binding and tyrosine phosphorylation of ER

Cytosol was separately prepared from 36 tumors (33 malignant and 3 benign tumors), and the ATP-dependent binding activation of ER was assayed (see Material and Methods). The results are listed in Table II. The table does not include a relapsed tumor that was studied separately (see Table III). In 7 samples, a large increase of estradiol binding sites over the basal level was induced by ATP (see values in bold in Table II). The increase ranged from 1 to 5 times the basal level of sites, with an average value of 3.4-fold. It is noteworthy that tumors showing an ATP-dependent increase of binding presented a low level of basal binding: on average, 37 fmoles of specific binding/mg protein (Table II, Fig. 1). This is significantly lower than the average from tumors not presenting this increase: 142 fmoles/mg protein (Table II, Fig. 1). The difference was reduced by incubation with ATP. When this was done, the average value increased from 37 to 127 fmoles/mg

TABLE IV – EFFECT OF ESTRADIOL ON HORMONE BINDING ACTIVATION AND TYROSINE PHOSPHORYLATION (INSERT PANEL) OF ER IN CYTOSOL OF HUMAN MAMMARY TUMORS

Tumor number	Specific estradiol binding sites[1]				ATP-activated binding sites[1]	
	−ATP		+ATP			
	−E2	+E2	−E2	+E2	−E2	+E2
4	38	38	73	245	35	207
10	74	74	87	165	13	91
12	24	24	10	89	-14	65



[1]Expressed as fmoles/mg cytosol protein. The assays of ATP-activated binding sites were performed using cytosols pre-incubated or not with estradiol (E2).–Insert panel: ER from cytosol of tumor number 10 was equilibrated or not with estradiol, incubated with ATP, then immunoprecipitated with anti-ER H222 MAb, resolved by SDS-PAGE and finally blotted with anti-phosphotyrosine MAb.

protein. The ATP dependence of hormone binding activation was studied by Scatchard plot analysis using cytosol from tumor 2 of Table II. Aliquots of cytosol were pre-equilibrated in the cold with different concentrations of [^{3}H]estradiol (from 0.08 to 8 nM) in the absence and in presence of excess cold hormone, then incubated at 15°C with or without ATP and finally assayed for [^{3}H]estradiol-specific binding activity. Data are presented in Figure 2*a*. Incubation with ATP induced a large increase in hormone binding to ER. This went from 53 fmoles/mg protein without ATP to 189 fmoles/mg protein with ATP. There was no change of affinity (K_d was about 1 nM in the absence or in the presence of ATP). An aliquot of the same cytosol was separately incubated with and without ATP. Proteins were immunoprecipitated with monoclonal anti-ER (H222) or control Ab and blotted with either anti-ER or anti-phosphotyrosine MAb. These findings are presented in the inserts of Figure 2*a:* equal amounts of immunoreactive ER are present in samples incubated without and with ATP; by incubation with ATP, the 67 kDa ER is phosphorylated on tyrosine. A doublet is seen at about 67 kDa. Also, tyrosine-phosphorylated ER in whole vanadate-treated MCF-7 cells migrated as a doublet (Auricchio *et al.,* 1995). The same tumor was employed to study the stability of the activity responsible for ATP-dependent hormone binding activation. The activity declined with a half-life of about 12 days (Fig. 2*b*).

Interestingly, ATP-dependent activation of estradiol binding, absent in a primary tumor (listed as number 3 in Table II), was present in the relapsed tumor (Table III). High expression of c-*erbB-2* was detected by Western blot analysis of protein from the particulate fraction of the relapsed tumor (see insert of Table III). Overexpression of c-*erbB-2* is associated with a poor clinical outcome in some human breast adenocarcinomas.

Effect of estradiol on the ATP-dependent activation of [^{3}H]estradiol binding and tyrosine phosphorylation of ER

Table II shows the increase of hormone binding sites in the presence of ATP when monitored using cytosol containing ER pre-incubated with a physiological concentration of estradiol. Omitting pre-incubation with estradiol, parallel incubations of cytosol from 3 of the 7 tumors reported in Table II were run. This resulted in a significant decrease or disappearance of ATP-activated binding sites (Table IV). Estradiol was also required for tyrosine phosphorylation of the 67 kDa ER in cytosol incubated in the presence of ATP and immunoprecipitated (insert of Table IV). It is noteworthy that in our experiments (see inserts of Fig. 2), the phosphorylated ER migrated as a 67 kDa protein like the classic ER. This indicates that the NHBER is different from truncated ER variants in human breast cancers with reduced receptor size (McGuire *et al.,* 1992).

Properties of the enzyme activating the hormone binding of the ER

The findings presented above led us to question whether altered ER PTK was present in this group of tumors. The observation that the same procedure efficiently purifies PTK from cytosol of bovine uterus and human mammary cancer (see Table I) suggests the presence of the same enzyme. This indication is reinforced by the presence of a 67 kDa protein in the enzyme preparation from tumors (see insert of Table I) and the dependence of the activity of the enzyme purified from tumors on the estradiol–receptor complex. Both properties, molecular weight under denaturating conditions and estradiol dependence of the activity, are in fact shared by the uterus enzyme (Castoria *et al.,* 1993). In addition, it is noteworthy that the enzyme from both sources interacts with calmodulin in the presence of Ca^{2+} and is stimulated by Ca^{2+}-calmodulin. In fact, the cancer enzyme activity is routinely assayed in the presence of 0.8 mM Ca^{2+} and 0.6 μM calmodulin (Castoria *et al.,* 1993). In the presence of 10 μM calmidazolium, which inhibits calmodulin-sensitive enzymes (Nicotera *et al.,* 1989), or 1 mM EGTA, which is a Ca^{2+} chelator, the activity is reduced to about a third of the activity assayed under routine conditions (not shown).

A "cold assay" based on blot analysis with anti-phosphotyrosine antibodies was employed to further study the estradiol–receptor complex dependence of the kinase using actin as a substrate. Before doing so, tyrosine phosphorylation of actin, ER and the protein carrier (soybean trypsin inhibitor) used during the acid precipitation of the assay mixture was analyzed

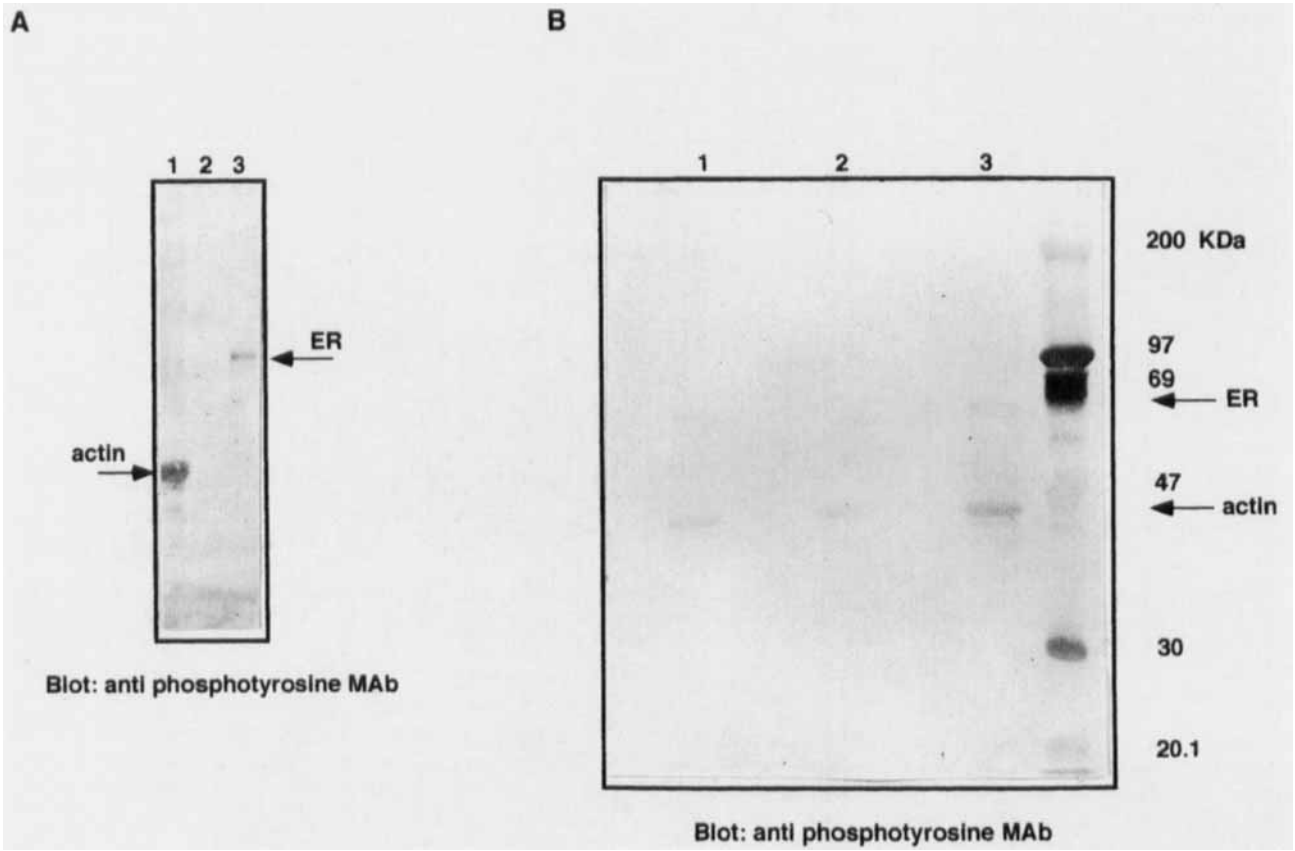


FIGURE 3 – Actin tyrosine phosphorylation by PTK purified from human mammary cancer. *(a)* Blot with anti-phosphotyrosine MAb of: actin (lane 1); electrophoretically purified soybean trypsin inhibitor used as a carrier (10 μg) for TCA precipitation of the ER (lane 2); 800 fmoles of pure estradiol–receptor complex precipitated by TCA in the presence of carrier (lane 3). Prestained molecular weight markers were co-electrophoresed and transferred in parallel (not shown). *(b)* Actin was incubated with ER and PTK in the presence of ATP as reported in the Material and Methods section. Electrophoretically separated proteins were transferred to nitrocellulose and hybridized with anti-phosphotyrosine MAb. Lane 1, actin plus ER; lane 2, actin plus PTK; lane 3, actin plus ER and PTK. Prestained molecular weight standards were co-electrophoresed and transferred at the right of lane 3. The arrows indicate the 43 kDa and 67 kDa positions of actin and ER, respectively.

(Fig. 3*a*). The protein carrier (lane 2) did not interact with anti-phosphotyrosine antibodies, whereas actin (lane 1) and 67 kDa ER (lane 3) were tyrosine phosphorylated. Tyrosine phosphorylation of ER in different tissues and cells has been reported (Migliaccio *et al.,* 1986; Arnold *et al.,* 1995; Auricchio *et al.,* 1995; Pietras *et al.,* 1995). The present observation that ER is also phosphorylated on tyrosine in calf uterus indicates that this phosphorylation plays an important role in receptor function. Figure 3*b* shows actin phosphorylation by the kinase. In the absence of ER (lane 2, Fig. 3*b*), the kinase did not phosphorylate actin; there is no difference between lane 2 and lane 1. The latter shows actin incubated in the presence of ER and in the absence of the enzyme. When actin was incubated in the presence of both the estradiol–receptor complex and the kinase, tyrosine phosphorylation of actin clearly increased over the intrinsic level (lane 3, Fig. 3*b*). In the same lane, a 67 kDa tyrosine phosphorylated protein, likely the ER, was detected. In conclusion, the PTK activity assayed either as actin (Fig. 3) and ER (insert of Table IV) tyrosine phosphorylation or as estradiol binding activation (Tables I, IV) was estradiol–receptor complex dependent. Figure 4*a* shows that PTK, assayed as hormone binding activation of partially dephosphorylated and inactivated bovine ER, is immunoprecipitated from cytosols of bovine uterus or human mammary carcinoma by anti-cst.1 antibody. This antibody is raised against amino acids 527–533 of the carboxy-terminal domain of the 60 kDa c-*src* and interacts with c-*src* and different members of the *src*-related kinases (Courtneidge and Smith, 1984). The same activity was not precipitated by the 327 MAb, which is specific for c-*src* (Lipsich *et al.,* 1983). Interaction between anti-c-*src* antibodies and estradiol-dependent PTK was further investigated using PTK purified from both human mammary cancer and calf uterus. The anti-RRDP antibody is raised against amino acids 500–512 of the catalytic domain of c-*src* and, like the anti-cst.1 antibody, can recognize not only c-*src* but also c-*src*-related kinases. Figure 4*b* shows that the 67 kDa PTK purified from mammary cancer interacts in immunoblot with anti-RRDP antibodies but does not interact with control (rabbit) antibodies. This proves that PTK directly interacts with anti-c-*src* antibodies. It also shows that the 43 kDa protein co-purified with the cancer PTK and detected by silver nitrate staining of the gel (see insert of Table I) does not interact with anti-c-*src* antibody. Figure 4*c* shows that the bovine enzyme after interaction in immunoblot with anti-cst.1 and anti-RRDP antibodies produced 2 bands, migrating at 67 and 62 kDa, respectively. The bovine enzyme did not interact with 327 MAb in immunoblot. A renaturation experiment (not presented) showed that both proteins (67 and 62 kDa) have pI of 6.6 and are endowed with estradiol-dependent PTK activity. This indicates that the 62 kDa PTK is related to the 67 kDa enzyme; it could be either an active proteolytic product of the 67 kDa enzyme or a form lacking a post-transcriptional

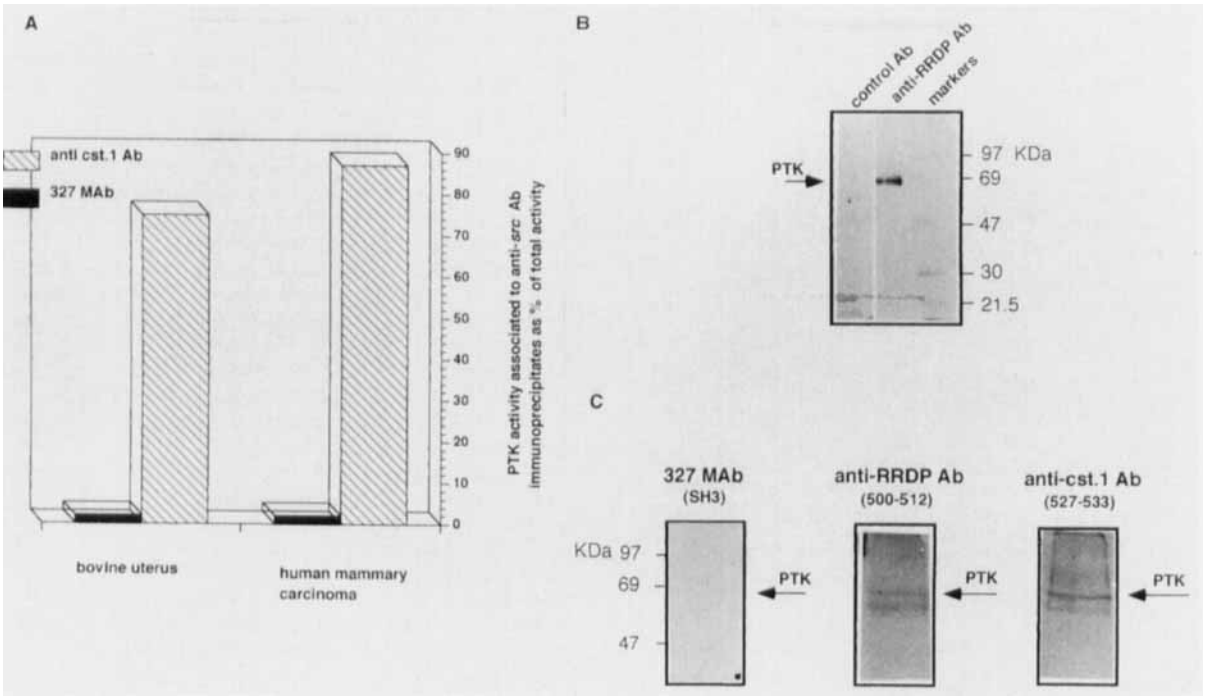


FIGURE 4 – Immunoprecipitation of PTK activity from bovine uterus and human mammary cancer cytosols by anti-c-*src* antibodies *(a)* and immunoblots of PTK from human mammary cancer *(b)* and bovine uterus *(c)* with anti-c-*src* antibodies. *(a)* PTK was precipitated from bovine uterus or human mammary cancer cytosols by control or anti-c-*src* antibodies as described. PTK activity was assayed before (total activity) and after immunoprecipitation using bovine PTP-inactivated ER as a substrate. The PTK activity specifically associated with anti-c-*src* Ab immunoprecipitates was expressed as percentage of the total activity. Dark bars, PTK activity associated with 327 MAb immunoprecipitates; shaded bars, PTK activity associated with anti-cst.1 Ab immunoprecipitates. *(b)* PTK purified from human mammary cancer was TCA-precipitated, reduced in Laemmli sample buffer and resolved in duplicate by SDS-PAGE. Electrophoretically separated proteins were submitted to immunoblot with either rabbit (control Ab lane) or anti-RRDP antibodies (anti-RRDP Ab lane). Prestained molecular weight markers were co-electrophoresed and transferred to the right of the anti-RRDP Ab lane. The arrow indicates the 67 kDa position of the purified PTK. *(c)* PTK isolated from bovine uterus was resolved in triplicate by SDS-PAGE. Proteins were transferred to filters and blotted with either 327 MAb, anti-RRDP Ab or anti-cst.1 Ab. The arrows indicate the 67 kDa position of the PTK. An additional 62 kDa immunoreactive band of PTK was detected in the central and right lanes (for details, see Results). Prestained molecular weight markers were co-electrophoresed and transferred in parallel (not shown).

modification present in the 67 kDa kinase. All together, the findings with anti-c-*src* antibodies further support the conclusion that bovine uterus PTK and human cancer PTK are the same enzyme. In addition, they indicate that the estradiol-dependent PTK has structural similarity with the carboxy-terminal and catalytic domain of the c-*src*-related kinases although it is not c-*src*. It is likely that it is a new member of the large c-*src*-related kinase family.

The ability of the 67 kDa PTK from mammary cancer to interact in blot with anti-RRDP antibody was used, in association with the PTK-activity assay, to study the distribution of the enzyme between soluble and particulate fractions. The results are reported in Figure 5. The specific activity of the enzyme is 4.4 times higher in the soluble fraction than in the particulate fraction. The 67 kDa band of the protein interacting with anti-RRDP antibody is also more prevalent in the soluble fraction than in the particulate fraction. The opposite is true for a protein with m.w. of about 57 kDa, likely a member of the *src* kinase family, which is more prominent in the particulate fraction than in the soluble fraction.

DISCUSSION

We report that NHBER is present in human mammary cancers. Others have observed that incubation with ATP of a cytosol pooled from several human mammary cancers caused an increase of estradiol-specific binding (Lahoti *et al.,* 1990). It is possible that this increase was due to the presence of NHBER. NHBER is converted to hormone binding receptor *in vitro* by an estradiol-dependent PTK (Castoria *et al.,* 1993). This suggests that the presence of NHBER is due to unbalanced phosphorylation of the 67 kDa receptor. In fact, experiments with partially (Migliaccio *et al.,* 1984, 1989; Auricchio *et al.,* 1987) or highly purified estradiol-dependent PTK (Castoria *et al.,* 1993) showed that ER tyrosine phosphorylation confers hormone binding to ER either synthesized by reticulocyte lysate or purified from uterus and pre-dephosphorylated by a nuclear PTP. The tyrosine residue whose phosphorylation confers hormone binding is tyrosine 537. We have identified this residue by site-directed mutagenesis (Castoria *et al.,* 1993). The same residue has been found to be phosphorylated in MCF-7 cells (Arnold *et al.,* 1995). More recently, the association between increases in tyrosine phosphorylation of ER and hormone binding to ER was observed in vanadate-treated MCF-7 cells (Auricchio *et al.,* 1995). Vanadate is a phosphotyrosine-phosphatase-specific inhibitor; its positive effect on hormone binding in whole cells indicates the presence of NHBER in these cells derived from mammary cancer cells and corroborates the physiological role of a nuclear phosphatase on estradiol binding of the receptor (Auricchio *et al.,* 1981). On the basis of these *in vitro* and *in vivo* experiments, our current findings suggest that the 67 kDa NHBER in mammary tumors is due to altered phosphorylation. How this occurs is unknown. Although it would be expected that NHBER associates with either reduction of the PTK activity or reduction of the ER dependence of this enzyme, a different

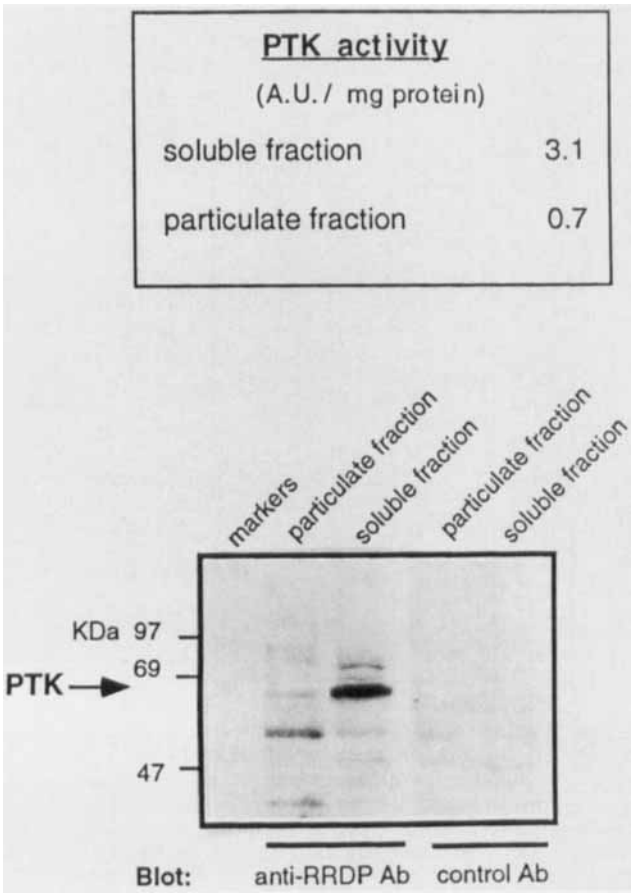


FIGURE 5 – Distribution of PTK from human mammary cancer between soluble and particulate fractions. *(Upper panel)* PTK activity from soluble and particulate fractions was assayed using bovine PTP-inactivated ER as a substrate. The enzymic activity was expressed as arbitrary units (A.U.)/mg of protein. *(Lower panel)* The soluble and particulate fractions from human mammary cancer were reduced in Laemmli sample buffer and resolved in duplicate by SDS-PAGE. Electrophoretically separated proteins were blotted with either rabbit (control Ab lanes) or anti-c-*src* (anti-RRDP) Ab (anti-RRDP Ab lanes). Prestained molecular weight markers were co-electrophoresed and transferred in parallel at the left of the filter. The arrow indicates the 67 kDa position.

mechanism must be responsible for the presence of NHBER in the analyzed human mammary tumors. In fact, the PTK activity is present in the cytosol of these tumors and is ER dependent. Intracellular mislocation of the PTK and/or ER preventing their correct interaction could be responsible for underphosphorylation of ER. Prevention of the correct interaction between PTK and ER by their association with other proteins in intact cells could also cause underphosphorylation of the receptor. We plan to analyze these as well as other mechanisms that could be responsible for the presence of NHBER.

The PTK from human mammary cancers shares numerous properties with the enzyme recently isolated from calf uterus (Castoria *et al.,* 1993): both enzymes are purified by the same procedure, show the same molecular weight under denaturating conditions, are stimulated by the estradiol–receptor complex, phosphorylate on tyrosine both ER (to which they confer hormone binding) and actin, interact with calmodulin in the presence of Ca^{2+} and are stimulated by Ca^{2+}-calmodulin. In addition, we now observe that both enzymes present a new and intriguing property. They interact with antibodies directed against both carboxy-terminal and catalytic domains of c-*src*. These antibodies associate not only with c-*src* but also with c-*src*-related kinases. Therefore, it is likely that the 67 kDa PTK is a new member of the *src* kinase family. The finding that such an enzyme is stimulated by the estradiol–receptor complex is strongly reminiscent of our recent observation that the same complex activates *in vivo* c-*src* (Migliaccio *et al.,* 1993). Therefore, it appears that a new property of this receptor, universally known as a transcriptional activator, is the activation of the mitogenic c-*src* and c-*src*-related kinases.

In conclusion, the present report shows that some human mammary cancers with low levels of hormone binding ER contain a large amount of NHBER, which is apparently different from the hitherto described abnormal estrogen receptors of human mammary cancers (McGuire *et al.,* 1992). In fact, the 67 kDa NHBER is converted into hormone binding receptor through tyrosine phosphorylation. This suggests that the defect responsible for the absence of hormone binding is not intrinsic to the receptor. Large amounts of NHBER could have some bearing on loss of hormone dependence, as indicated by its presence in hormone-independent mouse mammary tumors and its absence in hormone-dependent mouse mammary tumors (Migliaccio *et al.,* 1992), and on malignancy development, as suggested by its association with low levels of hormone binding ER and its appearance in a mammary cancer after relapse.

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