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dissertation entitled

Characterization of Protein Tyrosine Phosphatases in Human Breast Epithelial Cells Neoplastically Transformed by the neu Oncogene : The potential Role as a Tumor Suppressor.

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D degree in Pharmacology + Toxicology

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Date 6/29/93

CHARACTERIZATION OF PROTEIN TYROSINE PHOSPHATASES IN HUMAN BREAST EPITHELIAL CELLS NEOPLASTICALLY TRANSFORMED BY THE *NEU* ONCOGENE: THE POTENTIAL ROLE AS A TUMOR SUPPRESSOR.

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Toxicology

1993

ABSTRACT

CHARACTERIZATION OF PROTEIN TYROSINE PHOSPHATASES IN HUMAN BREAST EPITHELIAL CELLS NEOPLASTICALLY TRANSFORMED BY THE *NEU* ONCOGENE: THE POTENTIAL ROLE AS A TUMOR SUPPRESSOR

By

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Reversible phosphorylation/dephosphorylation of protein tyrosyl residues appears to be a key regulatory mechanism in the control of signal transduction, cell proliferation, differentiation and transformation; this metabolic process is modulated by the opposing activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases). While the role of PTKs has been examined extensively in human breast tumorigenesis, virtually nothing is known as to the role of PTPases in this tumorigenic process. To address this issue, two different approaches have been used: one approach was aimed at studying whether or not the introduction of an activated *neu* oncogene (a potent PTK) into an immortalized human breast epithelial cell line, could result in alterations of any PTPase expression. This approach led to the finding of a significant elevation in the expression of LAR and PTP 1B in three independent *neu* transformed human breast carcinoma cell lines in response to *neu* introduction. The level of *neu* expression , as well as the differential expression between P185neu and LAR and PTP 1B, directly correlated with tumorigenicity. Furthermore, elevated LAR-PTPase expression was observed in *neu* induced rat mammary carcinomas compared to carcinogen (7,12-dimethylbenzanthracene) induced rat mammary carcinomas. The second approach was to directly test the anti-oncogenic potential of LAR PTPase. To accomplish this, the full

length of human LAR cDNA, was constructed into an inducible expression vector. The level of LAR expression was modulated through transcription from a metallothionein (MT) promoter. When this LAR containing plasmid was introduced into a *neu* transformed human breast carcinoma cell line, a substantial increase in LAR expression in these cells was observed. This resulted in a change in the morphological appearence of these cells *in vitro*, and more importantly, a significant suppression of tumorigenicity of these cells inoculated into athymic nude mice. Thus, the relationship between the activities of $P185^{neu}$-PTK and LAR-PTPase, may be extremely important in human breast carcinogenesis.

THIS WORK IS DEDICATED TO:

My husband: Dajun Yang

My daughter and son: Alina Yang and Anthony Yang

My Parents: Gaosheng Zhai and Yanqun Xu

My country: China

ACKNOWLEDGEMENTS

I would like to express my appreciation to my co-advisors Dr. Clifford W. Welsch and Dr. Walter J. Esselman for their encouragement, financial support, friendship, guidance in science and scientific writing and many other ways of sharing through the years. I really enjoyed my mentor-mentee relationship! I also owe thanks to Dr. Richard Schwartz for his suggestions and advice as well as the other members of my graduate committee, Drs. Jay Goodman and John Thornburg for their advice and invaluable time.

A special debt of gratitude is extended to Dr. Michael Streuli and Dr. Justin McCormick for providing the anti-LAR monoclonal antibody and PTB-hyg vector, respectively. Without their generous assistance, much of my work would have been impossible. I also thank Drs. Kevin Walton and Jack Dixon for their assistance on the Southern blot analysis.

I would like to acknowledge all the past and the present members in Dr. Welsch's and Dr. Esselman's laboratories for their assistance, criticism and friendship. In particular, I would like to thank Dr. Hsun-Lang Chang and Mr. Howard Beittenmillier for their collaboration and technical advice. I am grateful to all of those who helped in one way or another during the course of this work.

Finally, I wish to thank my parents for their support and encouragement. I owe an unrepayable debt of appreciation to my husband, Dr. Dajun Yang, for his love, understanding, enduring support and sharing with me scientific thoughts as well as the care of our daughter Alina and our son Anthony. Without their support, the completion of this degree would not have been possible.

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INTRODUCTION

It is well known that protein tyrosine phosphorylation is a reversible reaction catalyzed by both protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTPase). Recent findings suggest that phosphoryltyrosine levels found within cells are the result of balance between the opposing activities of PTKs and PTPases (Fisher et al., 1991; Saito and Streuli, 1991). Although the phosphorylation on tyrosine residues within the cell occurs much less frequently than that on serine and threonine residues, tyrosine phosphorylation is a strictly controlled system involved in the regulation of key cellular activities including gene expression, signal transduction, proliferation and transformation.

A. HUMAN BREAST CANCER AND *neu* ONCOPROTEIN TYROSINE KINASE

The *neu* oncogene was first identified as a transforming oncogene in DNA from chemically (ethylnitrosourea) induced neuroblastomas in the rat (Schechter et al., 1985). The human *neu* proto-oncogene which encodes a 185 kDa transmembrane glycoprotein, $P185^{neu}$, shows extensive structural similarity to epidermal growth factor receptor (EGFR), a 170 kD transmembrane protein. Like EGFR, the *neu* protein possesses a hydrophobic transmembrane spanning sequence, a cytoplasmic portion with intrinsic protein tyrosine kinase (PTK) activity and the extracellular portion containing two

cysteine-rich clusters (repeat sequences) (Ullrich and Schlessinger, 1990). Several *neu* specific ligands with a variety of sizes have been identified. The identified ligands include 30 Kd (Lupu et al., 1990) and 45 Kd (Holmes et al., 1992) glycoproteins from conditioned medium of a human breast carcinoma cell line MDA-MB 231, a 44 Kd *neu* differentiation factor from the medium of a *ras* transformed fibroblast cell line (Peles et al., 1992; Yarden et al., 1991), and others isolated from transformed human T lymphocytes (Dobashi et al., 1991; Davis et al., 1991), macrophages (Tarakhovsky et al., 1991), and bovine kidney cells (Huang et al., 1990).

It appears that ligand-induced activation of the kinase domain in the P185^{neu} oncoprotein and its signaling potential are mediated by receptor dimerization (Hudziak et al., 1987). Ligand binding and subsequent conformational alteration of the extracellular domain including receptor dimerization, stabilizes interactions between adjacent cytoplasmic domains and leads to activation of the kinase function (Ullrich and Schlessinger, 1990). This dimer-mediated receptor activation allows heterodimer formation between structurally very similar receptors such as P185^{neu} and EGFR, resulting in the appearance of a very high affinity EGFR leading to an increase of P185^{neu} kinase activity for autophosphorylation and various cellular substrate phosphorylation (Wada et al., 1990; Qian et al., 1992). More recently, however, it has been demonstrated that EGF-induced heterodimerization of EGFR and P185^{neu} can promote either stimulatory or inhibitory influences on kinase activity, which lead to either activation or inhibition of the interaction of growth factor controlled cellular signaling (Spivak-Kroizman et al., 1992).

The normal function of the transmembrane domain is to anchor the receptor in the plane of the plasma membrane, thereby connecting the extracellular environment with internal compartments of the cell. The point mutations containing a substitution of Glu for Val at position 664 in transmembrane region of P185^{neu} result in constitutive receptor dimerization, thus leading to an activation of PTK, which is essential for the transforming potential of *neu* (Weiner et al., 1989). Recently, Cao et al. (1992) observed that the lateral position and rotational orientation of Glu664 did not correlate with transformation, but the primary structure in the vicinity of this Glu664 played a significant role in this activation. This suggests that the transmembrane domain, in particular the glycine-containing motif (amino acid 661-665) of the P185^{neu}, plays an active rather than a passive role in signal transduction.

The human *neu* proto-oncogene, also known as c-*erb*B-2 or HER-2, has been mapped to chromosome 17, region 21 (Coussens et al., 1985), and has been found to be amplified and/or overexpressed in approximately 30% of human primary breast cancer patients (Kraus et al., 1987; Slamon et al., 1987; Van de Vijver et al., 1987; Clark et al., 1991). The incidence and level of *neu* amplification has been found to remain consistent in matched primary and metastatic tumors from the same patient, suggesting that amplification is an early and possibly initiating event in human breast cancer (Lacroix et al., 1989).

The importance of the activated *neu* oncogene in experimental mammary carcinogenesis has previously been investigated using the transgenic mouse model (Muller et al., 1988; Bouchard et al., 1989). Little is known about its direct effects on the neoplastic transformation of human breast epithelial cells. Clark et al. (1988)

cotransfected v-Ha-*ras* and SV-40 large T antigen into an immortalized human mammary epithelial cell line, resulting in a strongly tumorigenic transformation. They claimed that complimentation between the two oncogenes (i.e., H-*ras* and large T antigen) was required to produce the fully carcinomatous phenotype. Recently, Pierce and her coworkers have demonstrated that the activated *neu* oncogene alone was sufficient for neoplastic transformation of immortalized human breast epithelial cells; such transfection resulted in progressively growing carcinomas in athymic nude mice. The data indicated that the overexpression of *neu* may directly contribute to the transformation of human breast epithelium (Pierce et al., 1991).

The activated form of $P185^{neu}$ exhibits a higher propensity to dimerize and an elevated tyrosine kinase activity (Bargmann and Weinberg 1988; Weiner et al., 1989). By using monoclonal antibodies (Scott et al., 1991,; Yarden et al., 1990) or chimeric *neu* proteins (Lehvaslaiho et al., 1989; Peles et al., 1991; Fagioli et al., 1991), it was possible to demonstrate that $P185^{neu}$-PTK could be stimulated and transmit growth regulatory biochemical signals. Cross-linking of $P185^{neu}$ with monoclonal antibody 4D5 on the $P185^{neu}$ overexpressing human breast carcinoma cell line, SK-BR-3, resulted in receptor phosphorylation and internalization, as well as increased intracellular levels of the second messengers: inositol triphosphates (IP_3) and diacylglycerol (DAG), and elevated expression of c-*fos* mRNA that directly regulated cell-cycle progression (Sarup et al., 1991; Scott et al., 1991). More interestingly, 4D5 stimulated rapid phosphorylation of $P185^{neu}$ and an associated 56-Kda phosphotyrosyl protein (P-Tyr56), with parallel elevation of PI4-kinase activity and production of the lipid substrate for

phospholipase-Cγ (PLCγ) (Scott et al., 1991). These data suggested that P-Tyr56 and PI4-kinase could represent specific substrates of P185^{neu}-PTK in its signaling cascade.

The transforming potential of *neu* oncogene is probably manifested via multiple genetic mechanisms as follows:

i) specific point mutation, a substitution of Glu for Val at position 664 in the transmembrane region (Bargmann et al., 1986; 1988; Weiner et al., 1989; Cao et al., 1992);

ii) extensive deletions of the noncatalytic portions in either the carboxyl or amino-termini (Di Fiore et al., 1987; Bargmann et al., 1988; Akiyama et al., 1991; Pierce et al., 1991). The proteolytic release of the extracellular domain is part of the mechanism by which the tyrosine kinase can become activated: a) The extracellular domain of P185^{neu} (105 Kd) was found to be shed in the human breast carcinoma cell line SK-BR-3 (Zabrecky et al., 1991); b) Expression of N-terminal truncated *neu* in NIH-3T3 cells resulted in a 10 fold greater transforming activity compared to the full length gene (Di Fiore et al., 1987). These data suggest that the remaining cell-associated cleavage product, probably represented an active and oncogenic form of the P185^{neu}.

iii) overexpression of the apparently normal proto-oncogene (human or rat) leading to transformation of NIH-3T3 fibroblasts (Di Fiore et al., 1987; Hudziak et al., 1987).

iv) ligand stimulation in the context of a chimeric *neu*-EGFR protein (Lehvaslaiho et al., 1989).

All these modes of oncogenic activation result in constitutive activation of *neu* intrinsic PTKs activity. Like other receptor linked PTKs, all autophosphorylation sites of $P185^{neu}$ are located in its carboxyl terminal tail (Margolis et al., 1989). Autophosphorylation of $P185^{neu}$ on tyrosine residues is absolutely required for intrinsic PTK activity (Sorkin et al., 1992) to trigger an efficient mitogenic response and high affinity PLCγ substrate coupling (Segatto et al., 1992). The EGFR/erbB-2 mutant bearing multiple Tyr to Phe substitutions at $P185^{neu}$ autophosphorylation sites was unable to deliver a sizeable mitogenic signal when activated by EGF treatment (Segatto et al., 1992). PLCγ is one of the known substrates for $P185^{neu}$-PTK and its catalytic activity is increased by tyrosine phosphorylation. Activation of PLCγ leads to rapid hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP2) to generate two second messengers, i.e. diacylglycerol (DAG) and inositol triphosphate (IP_3), which in turn activate protein kinase C (PKC) and mobilize intracellular calcium. The combined effect provides an internal stimulus for unregulated cell proliferation and neoplastic transformation (Ullrich and Schlessinger, 1990; Peles et al., 1991; Rhee et al., 1992). Replacement of the consensus lysine residue (Lys753 to Met) of the ATP binding site within the PTK domain completely abolished *neu* transforming ability (Akiyama et al., 1991). These results suggest that the mechanism of cellular neoplastic transformation by the *neu* oncoprotein involves tyrosine phosphorylation and activation of the PLCγ mediated phosphatidylinositol signalling pathway (Peles et al., 1991).

A number of studies have provided evidence that PLCγ activation is one of the critical elements in the mitogenic signaling pathway in human breast tumorigenesis. First, higher levels of tyrosine phosphorylated PLCγ proteins were detectable in the

majority of primary human breast carcinoma samples tested (18/21), compared to normal breast tissues (Arteaga et al., 1991). Second, tyrosine phosphorylation of PLCγ was greatly increased in human breast carcinoma cell lines as the result of EGF stimulation (Soderquist et al., 1992; Jallal et al., 1992). Third, it has been found that P185^{neu} formed a complex with PLCγ (Jallal et al., 1992). In fact, Peles et al. have demonstrated that the oncogenic forms of *neu* tyrosine kinase are permanently coupled to PLCγ, in which the tyrosine residues are constitutively phosphorylated. As tyrosine phosphorylated PLCγ mediates accelerated turnover of PI, protein kinase C and Ca^{++} fluxes are constitutively activated. This may in turn result in accelerated cellular proliferation, which is characteristic of the transforming phenotype (Peles 1991). Fourth, SH2 domains of PLCγ were able to enhance substrate phosphorylation by EGFR (Rotin et al., 1992a) and prevented tyrosine dephosphorylation of EGFR by PTPases (Rotin et al., 1992b). This suggests that SH2 domains of PLCγ could play a crucial role in P185^{neu} activation, because of its extensive structural similarity with EGFR.

In view of the role of *neu* oncogene in human breast carcinomas, it is important to note that *neu* gene amplification and overexpression have been found to be highly correlated with poor prognostic factors by using multivariate statistical analysis (Pierce et al., 1991; Van de Vijver et al., 1991). These factors include large tumor size, lymph node positivity, a higher number of involved nodes, advanced stage, steroid receptor negativity, aberrant DNA content and a higher rate of cell proliferation (Slamon et al., 1987; 1989; Berger et al., 1988, Wright et al., 1989, Thor et al., 1989; Ro et al., 1989; Tandon et al., 1989; King et al., 1989; Borg et al., 1991; Marx et al., 1990). Overexpression of *neu* is also strongly associated with early recurrence and death among

axillary lymph node positive patients (Borg et al., 1991). More recently, NIH 3T3 cells transformed by the activated rat *neu* oncogene have been shown to exhibit metastatic properties both *in vitro* and *in vivo* (Yu and Hung, 1991), indicating that *neu* oncogene expression is sufficient for the induction of metastasis in this cell line. Furthermore, human breast carcinomas overexpressing the *erb*B2 gene were resistent to certain cytotoxic drugs, and amplified expression of *neu* induced resistance of NIH 3T3 cells to tumor necrosis factor α (TNFα) and active macrophages (Hudziak et al., 1988). Thus, *neu* may potentiate tumorigenesis by inducing tumor cell resistance to host defense mechanisms. These findings suggest that amplification and/or overexpression of the *neu* proto-oncogene, and the consequent continued over- phosphorylation of tyrosine residues in cellular proteins, is an important and critical factor in the initiation of the growth and progression of human breast carcinomas.

B. PROTEIN TYROSINE PHOSPHATASES (PTPases): A DIVERSE FAMILY OF INTRACELLULAR AND TRANSMEMBRANE ENZYMES

1. Characterization of PTPases PTP1B, the first recognized member of PTPase gene family, was isolated in homogeneous form from the soluble and particulate fractions of human placenta by Tonks and his collaborators in 1988 (Tonks et al., 1988). PTP1B is active as a monomeric catalytic subunit of 321 amino acids, which is truncated product from the C-terminal. Intact PTP1B should contain 431 amino acids as predicted from the cDNA sequence. The gene coding for PTP1B has been mapped to chromosome 20q13.1-13.2, a region containing other genes which encode factors implicated in tumorigenesis, including *src*, hck and plc1 (Brown-Shimer et al., 1990). Amino acid

sequence comparison demonstrated that PTP1B is structurally not similar to any other catalytic subunits of serine/threonine phosphatases (Charbonneau et al., 1989), but was found to be homologous to the cytoplasmic domain of the leukocyte common antigen (CD45) (Charbonneau et al., 1988). CD45 is a transmembrane protein which possesses intrinsic tyrosine phosphatase activity (Cool et al., 1989).

By using low stringency screening methods, PTPases have now been identified in many different eukaryotic cell types in a broad range of sizes. The diverse family contains both transmembrane glycoproteins and cytosolic proteins (Fisher et al., 1991; Saito and Streuli, 1991). Cool et al. (1989) isolated a cDNA clone, TCPTP, from a human T-cell library which encoded a protein with 415 amino acids and displayed 65% sequence identity with PTP1B. Interestingly, the sequence similarity between PTP1B and TCPTP is only in the NH_2-terminal 300 amino acids (*i.e.*, within catalytic domains), with the remaining C-terminal sequences being distinct. The full-length and C-terminal truncated forms of TCPTP are found in distinct subcellular fractions and display differential effects on cell division and actin assembly (Cool et al., 1990; 1992; Fisher et al., 1991; Frangioni et al., 1992). It is known that the last 19 residues of C-terminal segment are hydrophobic and appear to be critical for subcellular localization, substrate specificity and regulation of the enzyme (Fischer et al., 1991). PTP1C is another cytosolic soluble PTPase isolated from human breast carcinoma ZR-75-1 cDNA library (Shen et al., 1991). Surprisingly, this enzyme is different from other cytosolic PTPases, in that it possesses a large noncatalytic region at NH_2 terminus and contains two adjacent SH_2 domains. The SH_2 domains of PTP1C formed a high affinity complex with activated EGFR which had intrinsic PTK activity. The finding of the SH_2 domain in

PTP1C as well as other PTPases (Adachi et al., 1992; Freeman et al., 1992; Matthews et al., 1992; Yi et al., 1992; Rotin et al., 1992b; Plutzky et al., 1992) indicates the linkage of PTPase to growth factor-receptors (PTKs) for regulation of signal transduction (Shen et al., 1991). The sequence homology of cytoskeletal protein and cytosolic PTPases indicated its potential role in controlling cytoskeletal integrity (Yang and Tonks, 1991; Gu et al., 1991).

Non-receptor, cytosolic PTPases possessing a single catalytic PTPase domain have been identified in viral, bacterial, yeast and mammalian species. An important question as to the origin of these genes was raised by the discovery of PTPases in pathogenic bacteria *Yersinia* (Guan et al., 1990) and *vaccinia* virus (Guan et al., 1991). What is the role of PTPases in developing and maintaining the pathological state? The transmembrane receptor type PTPases have been cloned from mammalian species and from *Drosophila*. They typically contain a single hydrophobic transmembrane region and two tandemly repeated conserved cytoplasmic domains fused to a variety of extracellular domains with great diversity in length and structure: several of which are related to neural cell adhesion molecules (N-CAM) (Streuli et al., 1988; 1989; 1990; Krueger et al., 1990) and some are homologous to carbonic anhydrases (Krueger and Saito, 1992).

CD45 or leukocyte common antigen (LCA) is found only in hematopoietic cells and is comprised of a family of heavily glycosylated transmembrane proteins with variable isoforms resulting from alternative splicing. CD45 contains two tandem PTPase-like domains with intrinsic PTPase activity (Charbonneau et al., 1988; Tonks et al., 1990). CD45-mutant T cell lines failed to elicit phosphatidylinositol (PI) turnover, indicating that CD45 is essential in linking the TCR complex to PI metabolism (Koretzky

et al., 1990). In B cells, CD45 may be a component of the antigen receptor complex (Justement et al., 1991). Extensive studies have indicated that CD45 plays a critical role in both T and B lymphocyte signal transduction. Activation and proliferation in response to antigen stimulation requires the direct dephosphorylation of Tyr505 of $P56^{lck}$ proto-oncogene by CD45 (Fisher et al., 1991; Saito and Streuli, 1991). More recently, Desai et al. reported that a chimeric protein in which the extracellular and transmembrane domains of CD45 were replaced with those of the EGF receptor (EGFR), was able to restore TCR signaling in a CD45 deficient cell. This data demonstrated that the cytoplasmic domain of CD45 is necessary and sufficient for TCR signal transduction (Desai et al., 1993).

Leukocyte common antigen related protein (LAR) is a transmembrane molecule composed of a 1234 amino acid extracellular receptor-like region, a 24-amino acid transmembrane segment and a 623 amino acid cytoplasmic region containing two tandemly repeated PTPase domains 40% homologous to PTP1B. It was found that the LAR gene was expressed on endothelial and epithelial cells of a broad range of tissue and cell types including lung, breast, kidney, thymus, brain, intestine, muscles and different tumor cell lines (Fisher et al., 1991; Saito and Streuli, 1991; Streuli et al., 1988). Streuli et al. (1992) have demonstrated that the LAR protein is proteolytically cleaved within the cell to produce a mature cell surface structure containing two subunits, termed the LAR-P-subunit (PTPase) and LAR-E-subunit (LAR extracellular subunit). The LAR-E-subunit can be released from the cell surface particularly in high density cell cultures. This suggests that LAR PTPase activity may be regulated by either unknown specific ligands or by shedding of the E-subunit. The extracellular region of LAR is composed

of three immunoglobulin (Ig) like domains and eight fibronectin type III (FN-III) domains which resemble neural cell adhesion molecules (N-CAM) (Streuli et al., 1988). N-CAM is a family of proteins derived from alternative mRNA splicing resulting in highly homologous extracellular domains (NH_2-terminal) but different cytoplasmic domains (Edelman et al., 1985). N-CAM is found on various differentiated tissues but mainly in neurons and muscles. It facilitates cell adhesion by homophilic cell-cell interaction among Ig-like and FN-III domains (*i.e.*, binding to the same molecules expressed on the surface of other cells) (Edelman et al., 1985; Cunningham et al., 1987; Owens et al., 1987). Hence, LAR may also be a cell adhesion molecule.

The physiological function of LAR PTPase may involve in controlling cell growth and proliferation through cell-cell or cell-matrix interactions (Fisher et al., 1991, Saito et al., 1991), the loss of which could lead to unrestrained cell proliferation and transformation (Saito and Streuli, 1991). Supporting this hypothesis is the finding that the product of a colorectal tumor suppressor gene, DCC (deleted in colorectal cancer), which was shown to be frequently deleted in colorectal carcinomas, is structurally similar to the extracellular region of LAR (Fearon et al., 1990; Standbridge, 1990). DCC gene has been mapped to chromosome 18q, for which approximately 40% of human breast carcinomas showed allelic losses (Devilee et al., 1991). More recently, Streuli et al. (1992) have demonstrated that the LAR gene is located on human chromosome 1p32-33, which contains several candidate tumor suppressor genes. Altogether, the unique structure of LAR PTPase raises the possibility that it is an ideal candidate for tumor suppressor gene.

Krueger et al. (1990) isolated six novel human placenta cDNA clones that encode receptor-like PTPases by cross-hybridization to *Drosophila* DPTP cDNA probe and named them HPTPα, β, c, δ, e, and f. HPTPα (RPTPα or LRP) and HPTPe have much smaller external domains than the rest. Variable cDNA structurns suggest that alternative splicing takes place in the first tyrosine phosphatase domain of LRP (Matthews et al., 1990). The human LRP gene has been assigned to chromosome 20p13 (Kaplan et al., 1990; Jirik et al., 1990). LRP and HPTPe are broadly distributed and mainly expressed in brain (Kaplan et al., 1990). They probably have a general function in signal transduction.

HPTPβ is unique and has only one PTPase domain on its cytoplasmic region and 16 repeated FN-III domains on the extracellular region. Most interestingly, the gene coded for RPTPγ has been localized to chromosome 3p21, and this PTPr allele was lost in approximately 50% of tumor samples examined (Laforgia et al., 1991). Such observations support the concept that certain RPTPases may be candidates for tumor suppressor gene products.

2. Structural and functional relationship of R-linked PTPases and the interactions between PTKs and PTPases The structural diversity of PTPases indicates that the PTPase gene family may be similar in complexity to that of the PTK multigene family (Kaplan et al., 1990; Hunter, 1989). Although the size of each PTPase domain is about 300 amino acids (AA), significant amino acid sequence similarity is seen only within the core region of about 250 AA. Krueger et al. (1990) compared the AA sequences of the core regions of 12 PTPases and found that 42 AA positions within the core region were totally invariant. PTPases, like PTKs, usually possess a consensus AA sequence in the

catalytically active domains. The most notable cluster in the stretch of AA within the consensus sequence is GPMVVHCSAGVGRTG, which surrounds the critical cysteine residue. It has been shown that this stretch constitutes an important portion of the catalytic sites of PTPases by site-directed mutagenesis (Streuli et al., 1990). The data indicates that the catalytic domains of all PTPases share significant amino acid homology and evolutionary conservation (Fisher et al., 1991; Krueger et al., 1990).

All receptor-linked PTPases characterized so far contain two PTP1B-like domains and all PTP1B-like sequences have two cysteine residues in the same relative positions (*i.e.*, within the highly conserved cluster) (Krueger et al., 1990; Streuli et al., 1990). The functional importance of two domains remains unclear. There are three possibilities: a) both domains are catalytically active and behave in a cooperative manner; b) both domains are catalytically active but have different substrate specificities; c) only one domain has enzymatic activity, while the other is regulatory in function. Since there is more constraint against sequence divergence in domain 1 than in domain 2, it may be that only the first domain has enzymatic activity (Laforgia et al., 1991; Pot et al., 1991). In fact, Streuli et al. demonstrated that the first domains of both LCA and LAR have enzymatic activity and that one cysteine residue is absolutely required for activity. In contrast, the second PTPase-like domains do not have detectable catalytic activity but sequences within the second domains influence substrate specificity and activity (Streuli et al., 1990; Itoh et al., 1992). In addition, a deletion (LAR: 1275-1311) which is upstream of the core sequence of LAR, completely abolished its PTPase activity (Streuli et al., 1990). On the other hand, using molecular cloning techniques, Wang et al. (1991) have shown that both α PTP domain 1 and domain 2 of HPTPα were enzymatically

active but differed in substrate specificity and responses to effectors. Inactivation of domain 1 may suppress domain 2 activity, suggesting that the interdependence between the two domains is important for PTPase function in signal transduction.

A variety of compounds has been shown to modulate PTPase activity in both a positive or negative manner. Orthovanadate is one of the most potent inhibitors of PTPases (Gordon et al., 1991; Pot et al., 1991; Hecht et al., 1992; Itoh et al., 1992; Tahiri-Jouti et al., 1992). The fact that thiol reducing agents are necessary for enzymatic activity suggests that cysteines play important roles in enzyme structure and function (Tonks et al., 1988). Three sulfhydryl agents (N-ethylmaleimide, p-(hydroxymecuric)benzoate and iodoacetate) inhibited rat LAR PTPase activity irreversibly (Pot et al., 1991), whereas the loss of PTPase activity caused by diamide is reversed by 2-mercaptoethanol or EGF in fibroblasts (Monteiro et al., 1991). It is important to note that both human and rat LAR PTPase activity is not inhibited by the divalent zinc, even at high concentration (1mM) *in vitro*, a characteristic which distinguishes LAR from other PTPases (Itoh et al., 1992; Pot et al., 1991; Wang et al., 1992). These results indicate that, despite the high degree of sequence conservation in the PTPase domains, these enzymes have different specific activities and respond differently to various modulators.

Since the level of protein tyrosine phosphorylation of intracellular substrates is determined by the balance of PTK and PTPase activity, their roles in regulation of signal transduction, cell proliferation, differentiation and neoplastic transformation have become an extremely interesting area of study. Although the finding of SH2 domain in PTP1C indicated the linkage between PTPase and PTK (Shen et al., 1991), nothing is known

about the direct interaction between PTKs and PTPases. A dynamic and complicated interaction between PTPases and PTKs or ser/thr kinases are likely to be involved in signaling pathways. There are a number of possibilities for such interaction.

a) As substrates, receptor linked PTKs are dephosphorylated by PTPases.

b) As substrates of PTKs or other protein kinases, PTPases are phosphorylated.

c) Both PTPases and PTKs have effects on common substrates (i.e. affect the state of activity of downstream secondary enzymes).

Indeed, it has been demonstrated *in vitro* that a number of receptor linked PTKs, such as EGFR, IGF-I, PDGF and insulin receptor, are substrates of different PTPases. The autophosphorylated EGFR was dephosphorylated by PTP1B (Tappia et al., 1991; Hashimoto et al., 1992a), by TCPTP (Zander et al., 1991), by LAR and by LRP (Hashimoto et al., 1992b) *in vitro*. The activated EGFR was able to form high affinity complexes with SH2 domains of PTP1C (Shen et al., 1991). Dephosphorylation of IGF-I receptor on tyrosine residues by membrane associated PTPases resulted in either a decrease in its intrinsic activity (Peraldi et al., 1992) or marked reduction in its autophosphorylation and mitogenic responses (Mooney et al., 1992). Hashimoto et al. demonstrated that LAR rapidly deactivated insulin receptor kinase by dephosphorylation on the receptor regulatory domains (Hashimoto et al., 1992b). Interestingly, the pattern of dephosphorylation of these receptor PTKs by PTPases *in vitro* occurs in a sequential or ordered manner, indicating that features surrounding the dephosphorylation sites might contribute to substrate specificity in intact cells (Ramachandran et al., 1992). Transmembrane PTPases identified exhibit high affinity for substrates and high activities

in cells, suggesting that these enzymes are important *in vivo* in controlling or reversing auto-phosphorylating PTK-induced regulatory or signalling events.

Several pieces of evidence support the second possibility. Since many possible sites of phosphorylation exist within the cytoplasmic domain of rLAR, the ability of both protein kinase C (PKC) and $P43^{v\text{-}abl}$ PTK to phosphorylate rLAR in a time-dependent manner *in vitro*, highly suggested that LAR may be regulated *in vivo* by these or similar enzymes (Pot et al., 1991). The ability of rLAR to undergo auto- or trans-dephosphorylation on phosphotyrosine residues parallels the autophosphorylation ability of PTKs (Pot et al., 1991). Brautigan et al. have shown that both the stimulation of cAMP dependent kinase (PKA) and Ca^{++}/phospholipid dependent PKC, and the inhibition of ser/thr phosphatases (especially type 2A), activated PTP1B activity in CV-1 monkey kidney epithelial cells. These data were consistent with observations in which phosphorylation of PTP 1B was elevated in TPA-stimulated HeLa cells (Flint et al., 1993). Phosphorylation of Ser/Thr residues in the regulatory subunit of PTP1B seemed sufficient to give full PTPase activity without dissociation or truncation of the catalytic subunit (Brautigan et al., 1991). Altogether, these data indicated that crosstalk between ser/thr kinases and PTPases may not alter basal levels of tyrosine phosphorylation, but modulate the response to stimuli. More recently, it has been shown that a mouse SH2-containing PTPase, Syp, was able to bind to autophosphorylated EGFR and was rapidly phosphorylated on tyrosine in EGF-stimulated cells. Also, Syp was constitutively phosphorylated on tyrosine in mouse fibroblast A31 cells transformed by *v-src* (Feng et al., 1993). These results support a model of regulation of PTPases by phosphorylation.

The cellular phosphorylation content may be regulated not only by PTK or PTPase activities, but also by SH2 domain containing proteins such as phospholipase Cγ (PLCγ). As evidence to support this, Rotin et al. have demonstrated recently that the tyrosine dephosphorylation of EGFR was inhibited by the SH2 domain of PLCγ, suggesting that the PTPases and the SH2 domains compete for the same tyrosine phosphorylation sites in the carboxy-terminal tail of EGFR (Rotin et al., 1992b). These data provide further support for the critical regulatory role of PLCγ and its SH2 domains in signal transduction and neoplastic transformation.

It is believed that the activity of cytoplasmic domains (PTPase domain) of receptor linked PTPases is regulated by specific unknown extracellular ligand, in a manner similar to the regulation of the growth factor receptor PTK activity (Hunter, 1989; Desai et al., 1993). The function and regulation of transmembrane PTPases have been difficult to study because of the paucity of information regarding ligands and *in vivo* substrates. Utilizing antibody crosslinking or chimeric protein models permits the study of regulation of PTPase without knowledge of the natural ligands. Indeed, Desai et al. recently reported that EGFR ligands functionally inactivated the EGFR-CD45 chimera in a manner that depended on dimerization of the chimeric protein. This indication resulted in the loss of TCR signaling. These data indicated that ligand-mediated regulation of receptor type PTPases may have mechanistic similarities with receptor PTKs. However, unlike receptor linked PTKs, which are activated by ligand binding, the family of transmembrane PTPases may, in general, be functionally inactivated by ligand binding (Tonks et al., 1990; Desai et al., 1993). The model for the mechanism of action of LAR proposed by Saito and Streuli (1990) stipulates that when a cell expressing LAR PTPase

encounters another cell carrying an unknown ligand, ligand binding to the extracellular domain of RPTPase might activate its cytoplasmic PTPase activity. The activated PTPase could, in turn, block the growth promoting effects of PTKs by either striping phosphate groups from the kinase themselves, thus shutting down one of the enzymes driving cell proliferation, or dephosphorylate cellular proteins that are also substrates for kinases. In addition, the preliminary results of Streuli et al. (1992) suggest yet another pathway for regulation of LAR PTPase, the proteolytic release of the extracellular subunit (similar to P185^{neu}).

In general, PTPases could be thought of as enzymes that serve to counteract the action of the PTKs resulting in the activation or inhibition of their target enzymes. It is possible too, that PTPases may act synergistically with PTKs (Fischer et al., 1991). This model of interaction between PTPase and PTK is supported by the observed activation of P56^{lck} PTK via CD45 dephosphorylation of the Tyr505 of Lck, an autophosphorylation site (Fischer et al., 1991; Saito and Streuli, 1991); and the activation of P34^{cdc2}, a critical component involved in cell cycle regulation, which starts the G2 to M transition by cdc25 PTPase dephosphorylation (Gautier et al., 1991; Dunphy et al., 1991). Thus, PTK or other kinase inhibition would be relieved by PTPase and the cellular response would be increased even though the level of substrate phosphorylation would decrease. On the other hand, a SH2 domain containing PTPase, PTP 1D, was found to be activated via phosphorylation on its tyrosine residue by a PTK. PTP 1D however, did not dephosphorylate receptor PTKs in vitro (Vogel et al., 1993). Thus, PTKs and PTPases do not simply oppose each other's action, rather they may work in concert to maintain a fine balance of effector activation needed for the regulation of cell

growth and differentiation. Therefore, the PTPases within the multigene PTPase family would vary in structure, mechanism of activation, regulation of activation, and interaction with specific PTKs.

3. Genes of PTPases - Tumor Suppressor Genes? Perhaps the most intriguing activity for PTPases is their possible role as tumor suppressors. It has known that reversible protein tyrosine phosphorylation is a fundamental mechanism for regulating diverse cellular processes including signal transduction, cell proliferation and neoplastic transformation. The constitutive activation of certain PTKs causes unregulated cell proliferation which is a very important component of oncogenesis. In fact, about one-third of all known oncogenes are PTKs, such as *neu*, *src*, *fms*, *abl*. Therefore, it is possible that certain PTPase genes are tumor suppressor genes (Fischer et al., 1991; Stanbridge, 1990; Sager et al., 1989). Conceptually, hyperphosphorylation of a key signal transduction protein driving neoplastic growth can occur either by deregulation or overexpression of PTK activity or by the loss of PTPase activity. Therefore, one could predict that overexpression of a PTPase counteracts oncogenic PTKs, either by conferring resistance to neoplastic transformation or by reversing a neoplastically transformed phenotype. On the other hand, loss or inactivation of both copies of a PTPase gene could result in constitutively increased tyrosine phosphorylation of particular cellular proteins, thus leading to tumorigenicity.

Among the multigenic PTPase family, LAR would appear to be an ideal candidate for a tumor suppressor gene because of its unique structure and broad tissue distribution (Fischer et al., 1991; Saito and Streuli, 1991; Streuli et al., 1988; 1990; Krueger et al., 1990). In contrast to LCA which is expressed only on hemopoietic lincage cells, LAR

is expressed on epithelial and endothelial cells of many different organ types, including lung, heart, kidney, thyroid, mammary gland, uterus, pancreas and nerve (Fischer et al., 1991; Streuli et al., 1990; 1992). Furthermore, the 72% homology of evolutionary conservation found between human LAR and its *Drosophila* homolog, DLAR, (Streuli et al., 1989) suggests that the function of LAR is fundamental to basic cell physiology. In addition, the overall structure of LAR, i.e., a cell adhesion molecule (N-CAM)-like structure on its extracellular subunit linked to a cytoplasmic PTPase domain, suggests that LAR may play an important role in the negative regulation of cell growth via cell-cell contact interaction. Further evidence to support the tumor suppressor gene concept is the finding that the product of a putative colorectal tumor suppressor gene, DCC (which was shown to be frequently deleted in colorectal carcinomas and perhaps in breast carcinomas) is structurally similar to the LAR E-subunit (Streuli et al., 1990). In addition, the human LAR gene has been mapped on chromosome 1p32-33 (Streuli et al., 1992), a region that contains a candidate breast carcinoma suppressor gene (Genuardi et al., 1989; Weinberg, 1991). This would suggest that LAR may be critical or at least very relevant to the development of human breast carcinoma.

The concept that PTPases may be tumor suppressor gene products is further supported by the following evidence:

a) Chromosomal localization. Chromosomal mapping of some PTPase genes is highly correlated with lesions known to be associated with various neoplasms. For example, the gene coded for human PTP1B has been mapped on chromosome 20q13.1-13.2 by *in situ* hybridization. This region contains several other genes such as *src*, *hck* and *plcl* which are involved in signal transduction (Brown-Shimer

et al., 1990). The human RPTPα gene has been mapped to chromosome 20pter-20q12, a region involved in translocations and deletions in myeloid disorders and neoplasms (Kaplan et al., 1990). RPTPγ gene is located on chromosome 3p21, a region which is also commonly deleted in renal and lung carcinomas (Laforgia et al., 1991).

b) Treatment with the PTPase inhibitor, vanadate, caused a phenotypic transformation of a fibroblast cell line (NRK) most probably due to increasing the amount of phosphotyrosine in these cells (Klarlund et al., 1985; Hunter, 1989).

c) When PTP1B or another cytosolic PTPase were introduced in NIH3T3 cells, the overexpression of this enzyme suppressed neoplastic transformation by the *neu* oncogene *in vivo* (Brown-Shimer et al., 1992), by the v-*src* oncogene *in vitro* (Woodford-Thomas et al., 1992) and by v-erbB oncogene (Ramponi et al., 1992).

d) Microinjection of PTP1B into Xenopus oocytes can reverse the mitogenic effects of insulin-stimulated tyrosine kinase activity (Cicirelli et al., 1990).

e) Overexpression of the truncated TC PTP resulted in approximately 50% reduction in a growth rate in baby hamster kidney cells (Cool et al., 1990).

f) A 37 Kd membrane associated nonreceptor type PTPase activity is significantly increased in Swiss 3T3 as well as normal fibroblast cells in response to density dependent contact inhibition (Pallen et al., 1991). This is consistent with the observation of Brautigan by using CV-1 monkey kidney epithelial cells (Brautigan et al., 1991).

These findings suggest that PTPases have anti-oncogenic potential. However, one hallmark of a tumor suppressor gene is that they are deleted in tumors in which their

inactivation contributes to the malignant phenotype. To support the concept that certain PTPases are tumor suppressor gene products, Laforgia *et al.* have demonstrated that loss of one allele of the human RPTPγ gene occurred in approximately one half of the tumor samples examined (Laforgia et al., 1991).

While certain PTPases are proposed to act as tumor suppressors, it is possible that other PTPases are involved in tumorigenesis in some other way, i.e., activation of PTPase actually stimulates the transforming process because some proto-oncogenes are known to be activated by tyrosine dephosphorylation, such as the *src* family tyrosine kinases, P56^{lck} and the cell cycle serine/threonine kinase P34^{cdc2}. It has been reported recently that overexpression of the receptor linked PTPa in rat embryo fibroblast, resulted in persistent activation of P60$^{c\text{-}src}$ and cell transformation (Zheng et al., 1992).

PTPases vary in structure, tissue distribution, mechanism of action and function even though they have highly homologous cytoplasmic PTP domains. There is no doubt that some of these PTPases (e.g. LAR, PTP1B and RPTPα, RPTPc), but not all, are excellent candidate tumor suppressor genes. The availability of cDNA clones for these PTPases and molecular cloning techniques will allow elucidation of the role of these PTPases, in particular receptor linked-LAR, in the initiation and growth of human breast carcinomas.

4. <u>The potential roles of PTPases in cell differentiation and programmed cell death.</u>

The potential role played by the changes in PTPase activity during differentiation is particularly interesting. Increased levels of certain PTPase activities have been

documented to be involved in morphological and functional differentiation in HL-60 cells (Frank DA et al., 1986; 1988; Buzzi et al., 1992), and other leukemia cell lines as well in response to growth inhibitory cytokines, such as IL-6 (Zafriri et al., 1993) or other maturational agents (PMA and DMSO) (Cohen et al., 1992; Butler et al., 1990; Buzzi et al., 1992). CD45 is one PTPase that plays a crucial role in hematopoietic cell differentiation (Buzzi et al., 1992). On the other hand, a rapid increased intracellular PTPase activity was observed during calcium induced keratinocyte differentiation (Zhao et al., 1992). More recently, den Hertog *et al.* (1993) demonstrated that activation of RPTγ (LRP) and other PTPases play an important role during neuronal differentiation (Aparicio et al., 1992). Taken together, these findings indicate that, like PTKs, PTPases indeed are involved in cell differentiation and development.

Programmed cell death (apoptosis) is an active cellular mechanism that is dependent on active participation of cellular components and could potentially be regulated by PTPases. Aberrant cell survival resulting from an inhibition of apoptosis could lead to oncogenesis. In contrast, induction of apoptosis in tumor cells could be used therapeutically (Williams et al., 1991). The morphological characteristics of apoptosis include nuclear condensation and DNA fragmentation. It has been demonstrated experimentally that expression of the *bcl2* oncogene product can inhibit apoptosis (William et al., 1991), whereas overexpression of wild type p53 tumor suppressor gene suppressed tumor growth (Shaw et al., 1992). Based on such observations, we could speculate that one of the mechanisms for p53 suppression of tumor growth may be the triggering of tumor cell death through apoptosis. Since certain genes that encode PTPases are good candidate tumor suppressor genes, it should not be

a surprising to find that activation of a PTPase may be associated with or even participate in apoptosis. In supporting this hypothesis, Bronte et al. (1993) found that addition of orthovanadate and phenylarsinooxide (PTPase inhibitors) or genistein (PTK inhibitor) both induced dose-dependent reduction in apoptosis without affecting cell viability. These preliminary data indicate that a complex interaction between PTKs and PTPase may be a crucial event in programmed cell death.

C. CELL CYCLE REGULATION

Normal eukaryotic cells exist in either a proliferative or a non-proliferative quiescent state. Proliferating cells progress through a chain of complex and tightly controlled events called the cell cycle. During one round of the cell cycle, a single parent cell prepares for and divides into two daughter cells. A typical eukaryotic cell cycle is composed of four major phases; G1, S, G2 and M phase.

A central control mechanism in the transition from interphase to mitosis is the activation of $P34^{cdc2}$-cyclin B complex called maturation-promoting factor (MPF). Recent studies have demonstrated that cdc25 is a specific protein phosphatase (PTPase), which by dephosphorylating tyrosine residues on $P34^{cdc2}$ directly activates $P34^{cdc2}$ (Gautier et al., 1991). Tonks and his coworkers showed that microinjection of purified PTP1B into Xenopus oocytes retarded maturation and meiotic cell division induced by insulin, and abolished insulin stimulation (Tonks et al., 1990). On the other hand, insulin increased mRNA expression of PTP1B in well-differentiated rat hepatoma cells, suggesting a potential mechanism for feedback desensitization of phosphotyrosine signalling through the insulin action pathway (Hashimoto et al., 1992c). In addition, by

using sensitive pattern-matching methods, they were able to detect a significant homology between cdc25 and the PTPase family including LAR and PTP1B (Gautier et al., 1991). Although it has been demonstrated that PTP 1B was associated with the endoplastic reticulum (Frangioni et al., 1992; Woodford-Thomas et al., 1992), increased phosphorylation of PTP 1B was seen to accompany the transition from G_2 to M phase of the cell cycle (Flint et al., 1993). This observation indicated that PTP 1B could be involved in controlling the structural changes in microtubules and the endoplastic reticulum that are associated with the cell cycle. More interestingly, overexpression of carboxyl-terminal truncated TCPTP in baby hamster kidney cells, resulted in cytokinetic failure and asynchronous nuclear division (Cool et al., 1992). These results suggested that certain PTPases (such as PTP 1B, TCPTP) other than cdc25 are directly or indirectly involved in the regulation of cell cycle progression.

Crissman and his coworkers compared the effects of staurosporine on the proliferation of non-transformed and neoplastically transformed cells and found that the kinase mediated regulation of G1 progression found in normal cells was lost as the result of neoplastic transformation (Crissman et al., 1991). This indicated that kinase mediated mechanisms play important roles in cell cycle regulation.

Many models for oncogenic transformation involve abnormal progression into the early stages of the cell cycle, such as observed with the oncogenes *mos*, *met*, *ras* and *src*. These oncogenes promote quiescent nondividing cells to enter the cell cycle at G1. In addition, altering the expression or activity of certain proto-oncogenes can result in inappropriate activation of MPF (M-phase promoting factor), which functions late in the cell cycle during the G2/M-phase transition. It is well known that many proto-oncogene

products including *src*, *abl*, *p53*, SV40 large T antigen and *mos* are substrates for $P34^{cdc2}$ phosphorylation of the catalytic subunit of MPF. The activation of these oncogenes results in activation of MPF, an essential molecule for initiation of mitosis in a variety of species (Freeman et al., 1991).

Little is known about the direct effects of *neu* (*erb*B2) proto-oncogene on cell cycle regulation. There is evidence that *erb*B2 amplification in human breast cancer is associated with a high rate of cell proliferation (Borg et al., 1991; Marx et al., 1990). In a study of 539 invasive primary breast carcinomas, Borg and his colleagues (1991) demonstrated that *erb*B2 amplification was strongly correlated to most known prognostic risk factors, and also correlated with an increased rate of cell proliferation and aberrant DNA content (large S phase fraction). However, a major issue unresolved in these studies is the mechanism by which *neu* regulates cell proliferation. It will be necessary to determine, therefore, whether or not *neu* amplification and overexpression results in higher carcinoma cell proliferation and whether or not such expression has a direct effect on certain specific regulators of cell cycle regulation. By comparing the cell cycle kinetics of *neu* and LAR transfected cells and their parental cells, one should be able to test the hypothesis that both *neu*-PTK and LAR PTPase are involved in the coupling of external signals to proteins such as MPF, a phenomena that is directly linked with cell cycle control.

D. SIGNIFICANCE OF THIS STUDY

There are now a number of studies which link overexpression and/or amplification of the *neu* proto-oncogene to neoplastic transformation and growth of human breast

carcinomas. It remains unclear how *neu*-PTK amplification and/or overexpression disturbs the balance between phosphorylation and dephosphorylation of cellular proteins and how this intricate balance influences oncogenesis on the molecular level. In addition, while much progress has been made in the identification of PTPase isoforms in recent years (Fischer et al., 1991; Saito and Streuli, 1991; Brautigan et al., 1992), little is known about their mechanisms of action or regulation. For example, literally nothing is known about the role of dephosphorylation which is catalyzed by *neu*-responsive protein tyrosine phosphatases (PTPases) especially when compared to what is known regarding the role of *neu*-PTK in human breast tumorigenesis. Hence, an understanding of the functions and interaction of *neu*-PTK and *neu*-responsive PTPases in normal and transformed cells will help to elucidate the role of these enzyme systems in normal cellular function as well as in the pathogenesis of human breast cancer.

Since LAR has an unique structure and broad tissue distribution, it is an ideal candidate for a tumor suppressor gene among the PTPase multigene family. The loss of this gene may confer a growth advantage on evolving human breast carcinomas cells. However, the critical role that LAR plays in normal and carcinomatous breast cellular processes has only recently been addressed and remains virtually unknown. Examination of the potential of LAR in reversing neoplastic transformation of *neu* transfected and/or over-expressing carcinoma cell lines, will allow one to evaluate and test the hypothesis. If the growth of *neu*-induced (or *neu* overexpressing) breast carcinomas can be suppressed or abrogated by the introduction of the LAR gene, LAR may have preventive or therapeutic potential. This may allow one to the design novel and effective

intervention protocols for the clinical treatment of human breast carcinomas and\or the prevention of this disease.

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CHAPTER II

INCREASED EXPRESSION OF SPECIFIC PROTEIN TYROSINE PHOSPHATASES IN HUMAN BREAST EPITHELIAL CELLS NEOPLASTICALLY TRANSFORMED BY THE *NEU* ONCOGENE

FOOTNOTES

Abbreviations: PTPase, protein tyrosine phosphatase; PTK, protein tyrosine kinase; RT-PCR, reverse transcription-PCR; LAR, leukocyte-common antigen related; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMBA, 7,12-dimethylbenzanthracene..

ABSTRACT

Protein tyrosine phosphorylation/dephosphorylation is a fundamental mechanism in the regulation of cell proliferation and neoplastic transformation; this metabolic process is modulated by the opposing activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases). While the role of PTK's has been examined extensively in human breast tumorigenesis, the role of PTPases in this process is virtually unknown. To address this issue, an activated *neu* oncogene was introduced into an immortalized non-tumorigenic human breast epithelial cell line (184B5). This resulted in a substantial increase in $P185^{neu}$ expression, which led to the formation of progressively growing carcinomas after such cells were inoculated into athymic nude mice. Importantly, a striking increase in the expression of specific PTPases, LAR and PTP1B, was observed in 3 independently *neu* transformed cell lines and their derived tumors. This elevation was verified at both the mRNA and protein levels. TC-PTP PTPase expression was only slightly increased in these *neu* transformed cells, and no expression of CD45 PTPase was observed. The level of *neu* expression, as well as the differential expression between $P185^{neu}$ and LAR/PTP1B, directly correlated with tumorigenicity. Furthermore, rat mammary carcinomas with elevated *neu* expression (*neu*-induced) also had sharply elevated LAR-PTPase expression when compared to rat mammary carcinomas with little or no *neu* expression (DMBA-induced); the level of expression of LAR PTPase was directly correlated with the level of *neu* expression.

Thus, our results provide the first evidence that, in human breast carcinoma cells and in rat mammary carcinomas that have an induced increase in *neu* expression, a consistent and substantial increase in the expression of specific PTPases occurs. The relationship between $P185^{neu}$-PTK expression and specific PTPase expression may play a critical role in human breast tumorigenesis.

INTRODUCTION

Reversible phosphorylation of protein tyrosyl residues is a fundamental mechanism for regulating diverse cellular processes including cell proliferation and neoplastic transformation. Modulation of protein phosphotyrosine is accomplished through the opposing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases) (1). The *neu* oncogene (c-*erbB*-2 or HER-2) is a member of the PTK class of oncogenes encoding a 185 kD transmembrane glycoprotein, $P185^{neu}$ (2). The transforming potential of the *neu* oncogene is manifested via multiple genetic mechanisms, including a point mutation within its transmembrane domain ($Val^{664} \rightarrow Glu^{664}$) (3,4), over-expression of the normal *neu* proto-oncogene product, and amino terminal truncation (5,6,7). All of these mechanisms result in constitutive activation of the *neu* intrinsic PTK activity, which is essential for transforming activity (2).

The human *neu* proto-oncogene has been found to be amplified and/or over-expressed in approximately 30% of primary human breast carcinomas (8, 9,10) and to be highly correlated with a poor prognosis (7,9,11,12). However, until very recently, the effects of induced *neu* over-expression in cultured human breast epithelial cells and the effects of such over-expression on the pathogenesis of these cells have remained unknown. Pierce and her coworkers (7) first reported that over-expression of normal $P185^{neu}$ alone was sufficient for neoplastic transformation of immortalized human breast epithelial cells. These findings strongly suggest that amplification and/or over-expression of the *neu* proto-oncogene, and the consequent continued over-phosphorylation of

tyrosine residues in cellular proteins, is an important and critical factor in the initiation, growth and/or progression of human breast carcinomas.

PTPases comprise a family of cytosolic nonreceptor and transmembrane receptor type proteins (1). LAR (13) and CD45 (14) represent receptor type PTPases whereas PTP1B (1,15) and TC-PTP (16) are cytosolic PTPases. PTPases have been identified in many different eukaryotic cell types and have been shown to participate in the various responses of cells to external stimuli (1). Recently, it was proposed that PTPases may have the potential to suppress the development and/or growth of tumor cells because of their ability to counteract the activity of PTKs (1). Evidence in support of this hypothesis includes the observation that the human PTP1B gene, when introduced into NIH 3T3 cells, suppressed neoplastic transformation by the *neu* (17) and the v-*src* oncogenes (18). In addition, Laforgia *et al.* have demonstrated that loss of one allele of the human hPTPγ gene occurred in approximately one-half of the human cancer samples examined (19).

There are now a number of studies which link over-expression and/or amplification of the *neu* proto-oncogene to neoplastic transformation and development of human breast carcinomas. However, it remains unclear how over-expression of the P185^{neu} oncoprotein disturbs the balance between phosphorylation and dephosphorylation of specific cellular proteins, and how this intricate balance influences oncogenesis on the molecular level. In addition, while much progress has been made in the identification of PTPases and in determining the structure and enzymatic properties of PTPases, little is known about their mechanisms of action or regulation. Virtually nothing is known about the role of dephosphorylation catalyzed by PTPases, especially when compared to

what is known regarding the role of P185^{neu}-PTK in human breast tumorigenesis. Hence, an understanding of the functions and interaction of P185^{neu}-PTK and PTPases in normal and transformed cells will help to elucidate the role of these enzyme systems in normal cellular functions as well as in the pathogenesis of human breast cancer.

MATERIALS AND METHODS

Cell lines. A benzopyrene induced, immortalized, but non-tumorigenic human breast epithelial cell line, 184B5, was obtained from Dr. M. Stampfer (Livermore Laboratory, Berkeley, CA) (20). 18-Rn1, 18-Rn2 and 18-Hn1 cell lines (preparation discussed below) and 184B5 cells were grown in MCDB170, prepared by mixing equal amounts of minimum essential medium (MEM) (GIBCO, Grand Island, NY) and keratinocyte basal medium, *i.e.* modified MCDB153 (Clonetics Corp., San Diego,CA), supplemented with epithelial growth factor (EGF, 10 ng/ml), insulin (10 μg/ml), transferrin (10 μg/ml),(Collaborative Research Inc., Bedford, MA), hydrocortisone (0.5 μg/ml, Sigma Chemical Co., St.Louis, MO) and Gentamicin (5 μg/ml, GIBCO). MCF-7 and SK-BR-3 human breast carcinoma cell lines were obtained from American Type Culture Collection (Bethesda, MD). MCF-7 and SK-BR-3 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (GIBCO). All cells were incubated at 37°C in 5% CO_2.

Animals. Female athymic nude mice (nu/nu) and female Sprague-Dawley rats were obtained from Harlan Sprague-Dawley, Indianapolis, IN. All animals were maintained according to the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals.

Retrovirus Infection. 18-Rn1 cells were derived by infecting 184B5 cells with a mutationally activated replication-defective amphotropic retrovirus vector, JR-*neu*, containing a single amino acid substitution (Val^{664} to Glu^{664}) in the transmembrane region. The rat *neu* oncogene was under the transcriptional control of the Moloney murine leukemia virus LTR. A neomycin resistance gene (neo^r) was used for selection.

The details for the construction of the JR-*neu* vector have been presented elsewhere (21). 18-Rn2 cells (obtained from Dr. C. Aylsworth, Michigan State University, E. Lansing MI) were derived by infecting 184B5 cells with a mutationally activated rat *neu* oncogene (identical to that above) utilizing the retrovirus expression vector pSV2neuT (22). 18-Hn1 cell line was derived by transfecting 184B5 cells with a mutationally activated human *neu* (*erbB*-2) oncogene (7) and was the generous gift of Dr. J. Pierce (National Cancer Institute, Bethesda, MD).

Induction of rat mammary carcinomas. The retrovirus vector described above (JR-*neu*) was infused once into the central duct of each mammary gland of 55 day old rats as described previously (21). To an additional group of rats, 7,12-dimethyl-benzanthracene (DMBA) (Upjohn Co., Kalamazoo, MI) was administered i.v. at a dose of 2 mg/100g body weight as described previously (23). Five weeks after *neu* infection or DMBA treatment, each rat had multiple mammary ductal carcinomas. The carcinomas were surgically excised, cleaned of connective tissues and subsequently prepared for RT-PCR analysis.

Oligonucleotide Primers. All oligonucleotide primers were synthesized in the Macromolecular Synthesis Facility, Michigan State University. The primers (including position in cDNA and the size of amplified fragment in bp) were as follows: 1. GAPDH (24), human CCATGGCACCGTCAAGGCTGAGAACG(228-253), CAATGCCAGC-CCCAGCGTCAAAGGT(964-939) 738bp, rat CCTGGCCAAGGTCATCCATG-ACAACTTTGG(501-530), CAATGCCAGCCCCAGCGTCAAAGGTG(928-903), 429bp; 2. LAR (13), human TCGAGCGCCTCAAAGCCAACG(4362-4382), GGCAGGCAC-CTCTGTGTGGCCG(5191-5170), 831bp, rat GAGGTGAACAAGCCCAAGAACCG-

CTAT(988-1014),CGGTTCTTGAACTTGTTGCAGGGCAGGTTG(1876-1847),890bp; 3. PTP1B (15), human CGGCCATTTACCAGGATATCCGACA(130-154), TCAGC-CCCATCCGAAACTTCCTC(839-817) 711bp, rat TCGATAAGGCTGGGAAC-TGGGCGGCTA(155-181), CCGTCTGGATGAGCCCCATGCGGAA(916-892), 763bp; 4. TC-PTP (16), human GCTGGCAGCCGCTGTACTTGGAAAT(110-134), ACTAC-AGTGGATCACCGCAGGCCCA(687-663) 579bp; 5. CD45(25), human GCATC-CCGCGGGTGTTCAGCAAGTT(2056-2080),TCCACTTTGTTCTCGGCTTCCAAGC-(2719-2695) 665bp; 6. *neu*, rat TGCCCCATCAACTGCACCCACTCCTGT(1907-1933), TCCAGGTAGCTCATCCCCTTGGCAATC(2541-2515), 636bp; Primers for human *neu* were identical to those previously reported (26). GAPDH was used as an internal control to insure that amplification had occurred in each sample and to correct for differences in the amount of mRNA in each sample (27).

RT-PCR analysis. Total RNA was isolated from cultured cells immediately before confluence and from freshly excised tumors derived from mice and rats (28), then RT-PCR was performed as previously described (29). Radioactive labeling was performed by addition of 1 μCi of (α-^{32}P)dCTP (Dupont/NEN Research Products, Wilmington, DE) to each PCR reaction mixture. At the end of every five PCR cycles, one reaction was stopped and the amplified products were analyzed by 8% native polyacrylamide gels and autoradiography. The amount of incorporated radioactivity of each gel band was determined by using a Betascope 603 blot analyzer (Betagen, Waltham, MA).

Immunofluorescence analysis. Cells were seeded at 1 x 10^4 cells per chamber (8 chamber slides; Nunc, Inc., Naperville, IL), incubated for 48 hours, then fixed with

100% methanol at -20°C. Anti-human c-*erbB*-2 monoclonal antibody was obtained from Oncogene Science, Inc. (Uniondale, NY), anti-human LAR mAb 11:1A (30) and anti PTP1B-Mab AE4-2J (17) were generous gifts from Dr. M. Streuli (Dana-Farber Cancer Institute, Boston, MA) and Dr. D. Hill (Applied Biotechnology, Cambridge, MA), respectively. The second antibody for c-*erbB*-2, LAR and PTP1B was FITC conjugated goat anti-mouse IgG (Sigma). The amount of *neu* oncoprotein, LAR and PTP1B proteins was quantitated by measuring the fluorescence intensities of the samples on a single-cell basis using an ACAS 570 interactive laser cytometer (Meridian Instruments, Inc., Okemos, MI).

Southern and Northern blot analysis. DNA was extracted using sodium dodecylsulfate-proteinase K and was digested with HindIII or BamHI, then run on 0.8% agarose gels and transferred to a nylon membrane (Schleicher & Schuell, Keene, NH). RNA was isolated using guanidine thiocyanate (28) and poly-A mRNA was isolated using a PolyATract mRNA Isolation System (Promega Co., Madison WI). mRNA was separated on a 1% MOPS-formaldehyde agarose gel and transferred to a nylon membrane (Gene Screen, Dupont/NEN Research Products). GAPDH (738bp) and *neu* (622bp) probes were prepared by PCR of cDNA of 18-Hn1 cells. The LAR (1306bp) and PTP1B (1273bp) probes were prepared by BamHI or HindIII digestion, respectively, of cloned cDNAs. Probes were labeled with $\alpha(^{32}P)$DCTP (Dupont/NEN Research Products) using a random primer labeling kit (United States Biochemical Co., Cleveland OH).

Flow cytometric DNA analysis. Bivariate flow cytometric measurement of BrdUrd incorporation and relative DNA content (propidium iodide staining) was

performed as previously described (31). Fluorescence intensity was determined with an Ortho cytofluorograph (Ortho Diagnostic Systems, Westwood, MA).

Tumorigenicity assay. Tumorigenicity of cell lines was determined by the ability of cells to form tumors in mature female athymic nude mice. 5×10^6 cells of each cell line were inoculated s.c. into athymic nude mice. Mice were observed at weekly intervals and tumor diameters were obtained by using a vernier caliper. The tumor volumes were calculated based on the formula $V = 4/3\pi(A+B/4)^3$ in which A and B are two perpendicular diameters obtained from each tumor.

RESULTS

Effect of *neu* introduction on 184B5 cell proliferation *in vitro*. After introducing the activated rat *neu* oncogene cDNA into the immortalized human breast epithelial cell line, 184B5, more than 1,000 G418 resistant colonies were observed on each 100 mm plate following a single exposure to 1 ml of JR-*neu* virus at 2 x 10^4 CFU/ml. These colonies were pooled and designated cell line 18-Rn1. As a consequence of *neu* introduction, 18-Rn1 and another *neu* transfected cell line, 18-Hn1, proliferated more rapidly compared to parental 184B5 cells. The population doubling times for 184B5, 18-Rn1 and 18-Hn1 were 28, 24 and 19 hrs, respectively. The *neu* transfection resulted in BrdUrd uptake of 30.3% and 38.9% in 18-Rn1 and 18-Hn1 cells, respectively, compared to 11.7% for 184B5 cells. This was confirmed using propidium iodide staining, in which the percent of cells in S phase of the cell cycle was 30.2% and 46.5% in 18-Rn1 and 18-Hn1 cells, respectively, compared to 13.5% for 184B5 cells. Thus, these data provide evidence that increased *neu* expression resulted in a substantial elevation of DNA synthesis and cell proliferation.

Effect of *neu* introduction on tumorigenicity of 184B5 cells in athymic nude mice. As a direct test of the transforming potential of the *neu* oncogene, the capability of *neu* transformed 184B5 cells (18-Hn1 and 18-Rn1) to form tumors in athymic nude mice was determined. Both 18-Rn1 and 18-Hn1 cells readily formed palpable tumors after s.c. inoculation (Fig. 1). 18-Rn1 cells formed tumors which initially grew progressively, followed by a period of slow growth; a number of these tumors ultimately regressed. 18-Hn1 cells formed rapidly growing progressive tumors; no evidence of

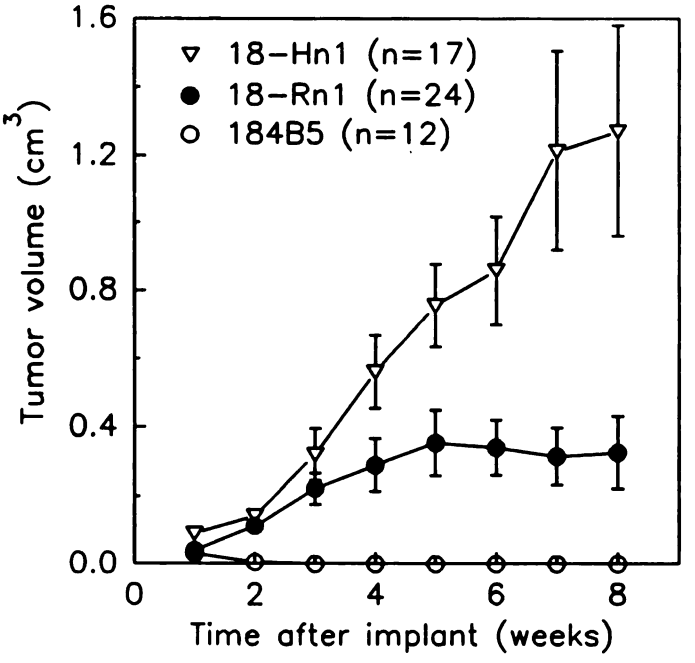

Fig. 1. Tumorigenicity of *neu* transformed 184B5 human breast epithelial cells. Tumor growth of *neu* transformed 18-Hn1 (-▽-) and 18-Rn1 (-●-) cells, and parental 184B5 (-○-) cells was measured after s.c. inoculation of these cells into athymic nude mice. The number in parentheses is the number of inoculation sites for 184B5 cells, or the number of tumors resulting from inoculation of 18-Hn1 and 18-Rn1 cells. Data points are the mean tumor volume ± SEM.

regression was observed in these tumors. Histological analysis revealed that all 18-Rn1 tumors exhibited many characteristics of human breast carcinomas, including a high degree of nuclear polymorphism, central necrosis and cysts. Histological characteristics of 18-Hn1 tumors were similar to 18-Rn1 tumors but lacking substantial cystic formation. No tumor development was observed from the parental 184B5 cell line.

Expression of *neu* and PTPases in 184B5 cells and in *neu* transformed 184B5 cells. Since virtually nothing is known about PTPases in either normal or neoplastic human breast epithelial cells, 4 different PTPases (LAR, PTP1B, TC-PTP and CD45) were studied by RT-PCR. Labeling with (α-^{32}P)dCTP was used to determine the RT-PCR amplification efficiency and to semi-quantitatively compare amplified PCR products. The amount of radioactive amplified product produced for each PTPase, GAPDH, and *neu* was measured every 5 PCR cycles by scanning dried polyacrylamide gels using a Betascope radioanalytic system (Fig. 2). This allowed determination of the range in which amplification proceeded with similar efficiency. Since amplification generally reached the plateau range at more than 25 cycles, the data used for comparative analysis was at 25 cycles of amplification. Thus it was possible to compare the initial amounts of mRNA template by comparing the amounts of amplified PCR products.

Based on this analysis, the expression of *neu* and 4 PTPases in 18-Rn1, 18-Hn1 and 18-Rn2 cells and the parental 184B5 cells were compared using the amount of GAPDH as an internal control. A summary of these data is shown in Table 1. In order to compare cell lines, the expression levels of *neu* and the PTPases were standardized relative to 184B5. *Neu* was elevated from 5 to 22 fold in the 3 independently

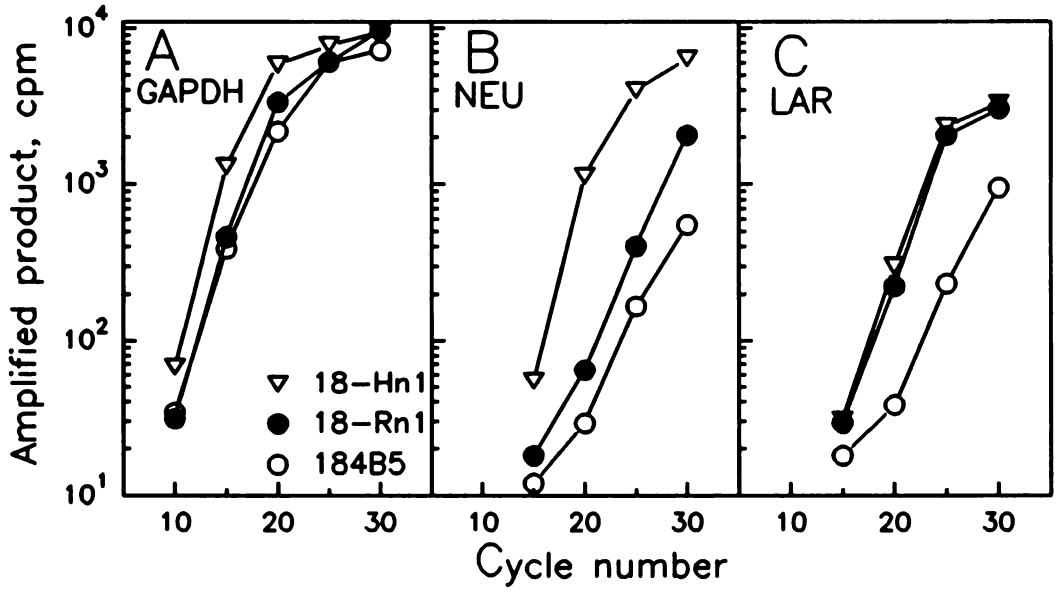

Fig. 2. Kinetics of RT-PCR amplification of GAPDH, *neu* and LAR in *neu* transformed 184B5 human breast epithelial cells (18-Rn1, 18-Hn1) and parental 184B5 cells. mRNA from 184B5 (-○-), 18-Rn1 (-●-) and 18-Hn1 (-▽-) cells was transcribed and cDNA was amplified for the indicated number of cycles. (α-^{32}P)dCTP radioactivity (cpm) incorporated into the amplified products is plotted against the number of PCR cycles. CPM were obtained by a Betascope 603 blot analyzer.

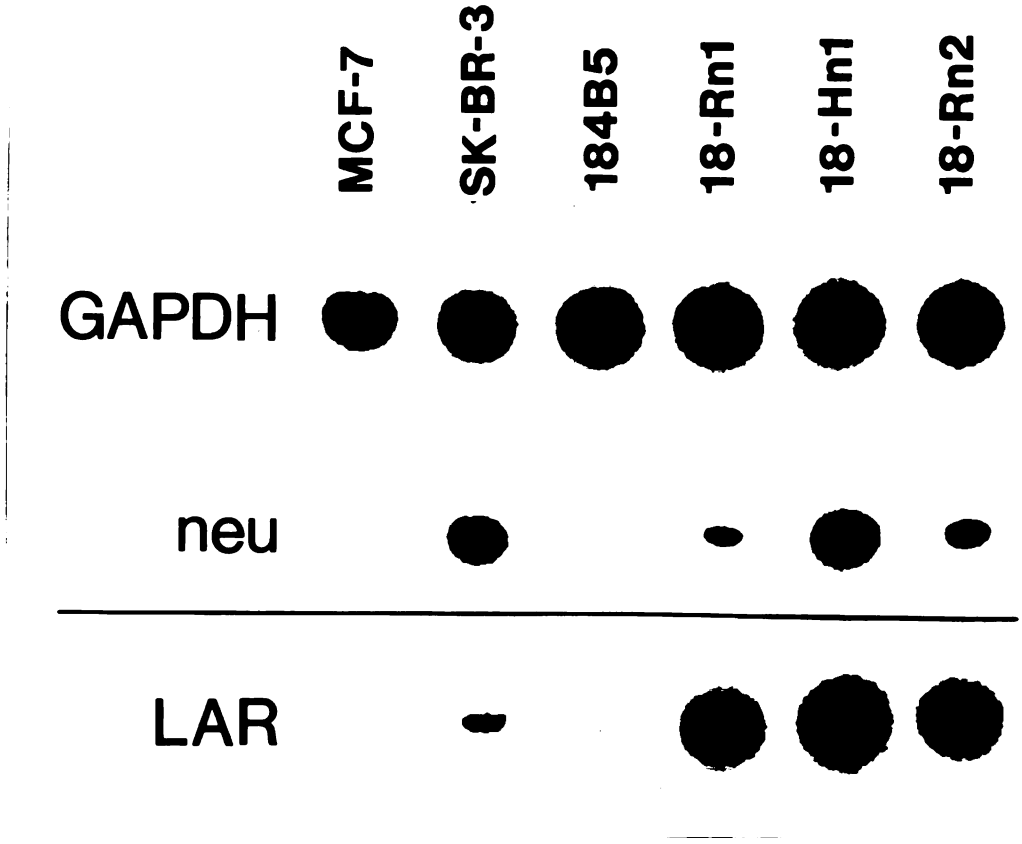

Fig. 3. Expression of GAPDH, *neu* and LAR specific RT-PCR products from *neu* transformed 184B5 human breast epithelial cells (18-Rn1, 18-Hn1, 18-Rn2) and parental 184B5 cells. MCF-7 and SK-BR-3 human breast carcinoma cells served as positive and negative controls. PCR was performed in the presence of (α-^{32}P)dCTP for 25 cycles and the gel separated products were analyzed using the Betascope blot analyzer. The sizes of the specific products are: GAPDH, 738 bp; *neu*, 266 bp; and LAR, 831 bp.

Table 1. Expression of *neu*, and LAR, PTP1B and TC-PTP in *neu* transformed 184B5 human breast epithelial cells and in SK-BR-3 and MCF-7 human breast carcinoma cell lines determined by RT-PCR analysis.*

Cells	Ratio of PCR amplified product to 184B5 (mean ± SEM)†			
	neu	LAR	PTP1B	TC-PTP
184B5	1	1	1	1
18-Rn1	5.9 ± 2.7	14.2 ± 4.9	7.4 ± 4.6	2.9 ± 1.5
18-Hn1	22.2 ± 1.9	13.6 ± 4.8	7.1 ± 2.5	5.4 ± 2.0
18-Rn2	5.0 ± 0.9	16.4 ± 5.1	8.6 ± 1.4	3.6 ± 0.7
SK-BR-3	23.1 ± 1.7	1.8 ± 0.2	1.1 ± 0.3	0.2 ± 0.1
MCF-7	2.1 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.2 ± 0.1

* Expression level of *neu* and PTPases were standardized to the expression of GAPDH.

† Expression levels shown are the mean of 3 to 4 individual experiments.

transformed 184B5 cell lines (18-Rn1, 18-Hn1 and 18-Rn2)(Table 1, Fig. 3). PTPase expression was increased in the 3 independently derived *neu* transformed 184B5 cell lines, to similar levels, even though the expression of *neu* was much higher in 18-Hn1 cells compared to 18-Rn1 and 18-Rn2 cells. Of the 4 PTPases examined, LAR expression increased the most ($\approx$15 fold), followed by PTP1B expression ($\approx$8 fold). TC-PTP expression was observed to increase slightly and CD45 was not expressed in these cells (data not shown). SK-BR-3 and MCF-7 are two well-known human breast carcinoma cell lines; substantial expression of PTPases was not observed in these cell lines despite the fact that one of these lines (SK-BR-3) has high *neu* expression.

In an effort to determine whether or not a correlation exists between *neu* and PTPase expression and tumor growth (in athymic nude mice), we subjected total cellular RNA from 18-Rn1 and 18-Hn1 tumors to RT-PCR analysis. As shown in Fig. 4, expression levels of *neu* were substantially higher in the faster growing, more progressive 18-Hn1 tumors than in the slower growing 18-Rn1 tumors (Fig. 4, lane 5-9 vs. lanes 2-3). LAR PTPase was observed to be elevated to about the same extent in both the rapidly growing tumors (18-Hn1) and the slower growing tumors (18-Rn1). PTP1B was elevated to a greater extent in 18-Hn1 tumors than in 18-Rn1 tumors. TC-PTP was elevated comparably, in both the 18-Rn1 and 18-Hn1 tumors. To assure that the mRNA expression of neu and PTPases detected using RT-PCR analysis infers the relative amount of mRNA in the cell lines studied, Northern blot analysis was performed. Since the mRNA expression of PTPases in these cell lines was extremely low, 500-1000 μg of total RNA from each cell line was required to generate sufficient mRNA to be detectable via

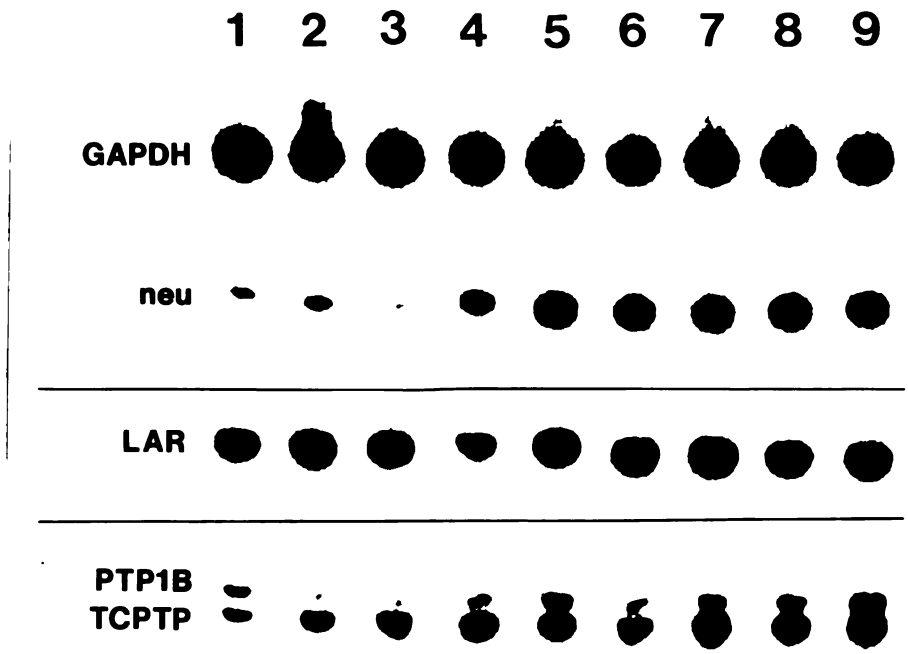

Fig. 4. Expression of GAPDH, *neu*, LAR, PTP1B and TC-PTP specific RT-PCR products from *neu* transformed 184B5 human breast epithelial cells (18-Rn1, 18-Hn1) and 18-Rn1 and 18-Hn1 human breast tumors. Lane 1, 18-Rn1 cells; lanes 2 and 3, 18-Rn1 tumors; lane 4, 18-Hn1 cells; lanes 5-9, 18-Hn1 tumors. PCR was performed in the presence of (α-^{32}P)dCTP for 25 cycles and the gel separated products were analyzed using the Betascope blot analyzer. The sizes of the specific products are: GAPDH, 738 bp; *neu*, 266 bp; LAR, 831 bp; PTP1B, 711 bp; and TC-PTP, 579 bp.

Northern blot. The results were consistent with our RT-PCR data, i.e. the mRNA expression of LAR and PTP1B was elevated in the *neu* transformed cell lines compared with the parental 184B5 cell line (data not shown).

Expression of *neu* and PTPases in *neu*- and DMBA-induced rat mammary carcinomas. The expression of *neu* and PTPases was compared in rat mammary carcinomas induced *in vivo* by *neu* infection or DMBA treatment (Fig. 5A and B). After RT-PCR analysis, *neu* expression was observed to be much higher in *neu*-induced rat mammary carcinomas than in DMBA-induced carcinomas. This shows that *neu* was successfully infected into rat mammary epithelial cells *in situ*. Importantly, LAR expression was also substantially higher in *neu*-induced rat mammary carcinomas compared to DMBA-induced carcinomas (Fig. 5B). Furthermore, there was a strong correlation between the magnitude of the expression of *neu* and LAR (Fig. 5B, insert). There was no observed difference in PTP1B expression when comparing *neu*- and DMBA-induced rat mammary carcinomas.

Expression of $P185^{neu}$, LAR and PTP1B protein in 184B5 cells and in *neu* transformed 184B5 cells. To assure that the relative levels of mRNA inferred by the above RT-PCR and Northern blot analysis were reflected in protein levels, immunofluorescent analysis was performed to determine $P185^{neu}$, LAR and PTP1B protein expression in 184B5, 18-Rn1 and 18-Hn1 cells and SK-BR-3 and MCF-7 cells. Using respective primary antibodies and FITC second antibodies, the relative amounts of $P185^{neu}$, LAR and PTP1B proteins in individual cells were determined as fluorescent units by using quantitative interactive laser cytometry. Our results show that $P185^{neu}$

Fig. 5. Expression of GAPDH, *neu*, LAR and PTP1B specific RT-PCR products from *neu*-induced and DMBA-induced rat mammary carcinomas. PCR was performed in the presence of (α-^{32}P)dCTP for 25 cycles. A. Autoradiogram of GAPDH, *neu*, LAR and PTP1B specific RT-PCR products. Each lane represents an individual tumor. The sizes of these products are: GAPDH, 429bp; *neu*, 636bp; LAR, 890bp and PTP1B, 763bp. B. Comparison of incorporated radioactivity (cpm) of each specific RT-PCR product measured using a Betascope blot analyzer. B, insert. Relationship between incorporated radioactivity (cpm) of LAR and neu specific RT-PCR products in individual *neu*-induced rat mammary carcinomas. Regression correlation coefficient of line equals 0.834.

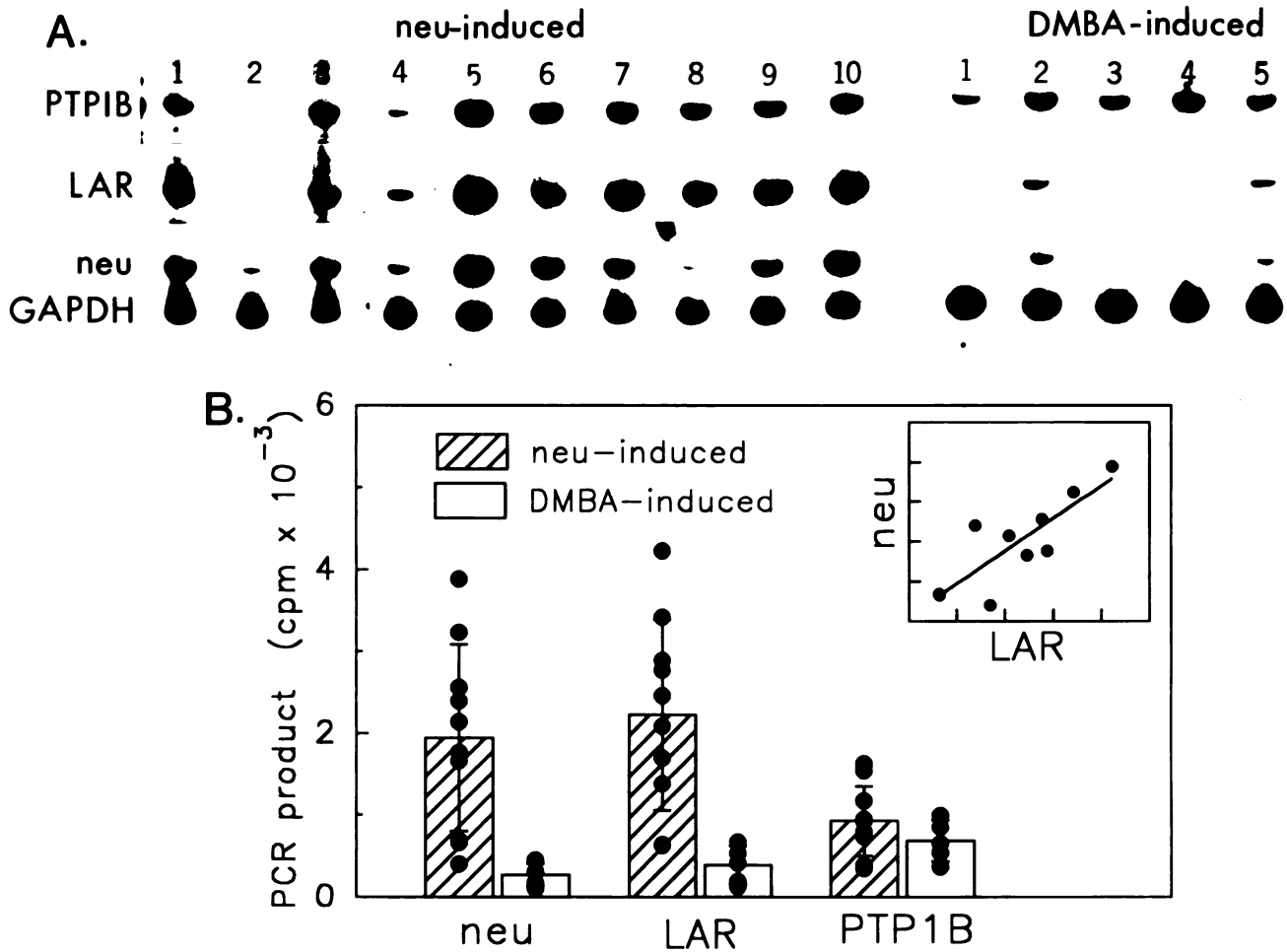
A.
neu-induced
DMBA-induced
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5
PTPIB
LAR
neu
GAPDH
B.
PCR product (cpm x 10^-3)
6
4
2
0
neu–induced
DMBA–induced
neu
LAR
neu
LAR
PTP1B

Table 2. Expression of proteins P185^{neu}, LAR and PTP1B in *neu* transformed 185B5 human breast epithelial cells and in SK-BR-3 and MCF-7 human breast carcinoma cell lines determined by immunofluorescence analysis.

Cells	Fluorescent units x 10^{-2} / cell (mean ± SEM)		
	P185^{neu}	LAR	PTP1B
184B5	195 ± 3 (170)*	489 ± 29 (114)	166 ± 1 (210)
18-Rn1	2,943 ± 59 (235)	4,641 ± 54 (181)	425 ± 6 (163)
18-Hn1	43,985 ± 376 (119)	5,947 ± 252 (114)	1,414 ± 20 (118)
SK-BR-3	6,469 ± 4 (73)	464 ± 4 (86)	296 ± 10 (73)
MCF-7	344 ± 7 (125)	354 ± 1 (65)	163 ± 8 (83)

* Number in parenthesis represent the number of cells analyzed.

protein was expressed an average of ≈15 fold and ≈225 fold higher in 18-Rn1 and 18-Hn1 cells, respectively, compared to 184B5 cells (Table 2). Levels of LAR protein were expressed an average of ≈9 fold and ≈12 fold higher in 18-Rn1 and 18-Hn1 cells, respectively, compared to 184B5 cells. Expression levels of PTP1B protein in 18-Rn1 and 18-Hn1 cells were, on average, ≈3 fold and ≈8 fold higher, respectively, compared to 184B5 cells. Of the two human breast carcinoma cell lines, SK-BR-3 expressed very high levels of P185^{neu} protein whereas MCF-7 cells expressed low levels of P185^{neu}, results which are in accord with literature reports (6,8). Both SK-BR-3 and MCF-7 cells expressed LAR and PTP1B proteins at relatively low levels.

Southern blot analysis. Southern blot analysis was performed to examine whether or not the increased expression of LAR and PTP1B resulted from gene amplification. The LAR and PTP1B genes were not found to be amplified among the six cell lines studied (Fig. 6, data shown for LAR only). In addition, the *neu* gene was found to be amplified in SK-BR-3 cells (positive control) in accord with literature reports (6,8) (data not shown).

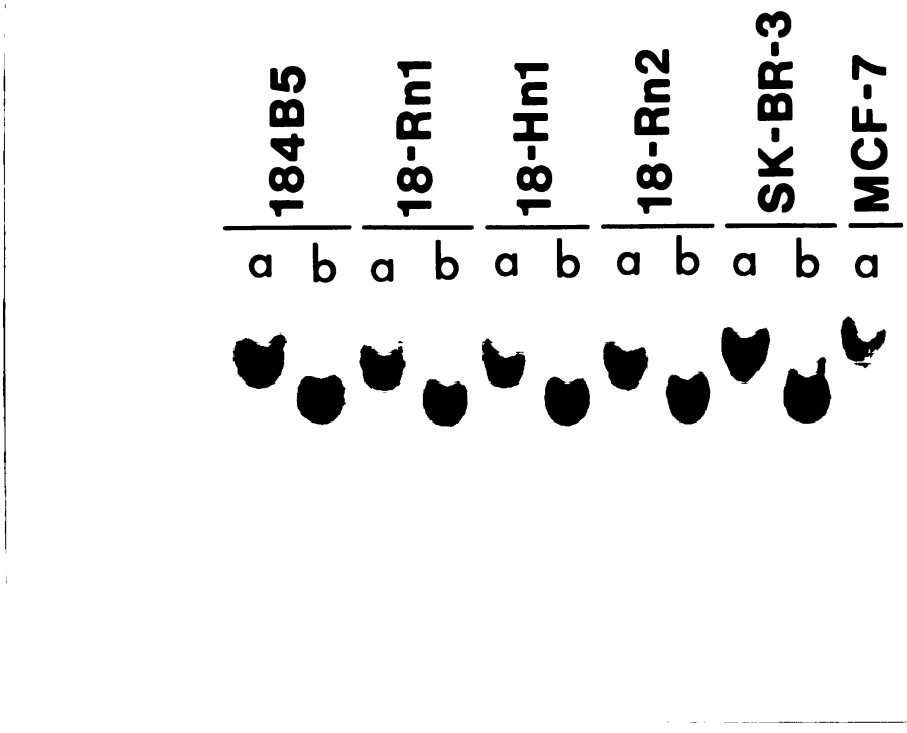

Fig. 6. Southern blot analysis of LAR in *neu* transformed 184B5 human breast epithelial cells. Isolated DNA was digested with either HindIII (lane a) or BamH1 (lane b) restriction enzymes. The Southern blot filter was hybridized with the ^{32}P-labeled specific LAR RT-PCR product.

DISCUSSION

We report herein the neoplastic transformation of an immortalized but non-tumorigenic human breast epithelial cell line (184B5) by introduction of a mutationally activated *neu* oncogene. We have demonstrated that over-expression of P185^{neu}, which was confirmed at both the mRNA level and at the protein level, is capable of conferring a neoplastic phenotype to these cells. The consequences of introduction of the activated *neu* oncogene into 184B5 cells included increased DNA synthesis, increased cell proliferation, and the formation of progressively growing carcinomas when inoculated into athymic nude mice. Inoculation of 18-Hn1 cells (high expression of P185^{neu}) into athymic nude mice resulted in tumors with a rapidly progressive growth phenotype, whereas 18-Rn1 cells (lower expression of P185^{neu}) resulted in carcinomas with a slower growth rate, a number of which eventually regressed. Thus, the level of expression of P185^{neu} was directly associated with increased tumorigenicity *in vivo*. Our results confirm the recent observations by Pierce and colleagues (7) who reported that introduction of a mutationally activated *neu* oncogene into 184B5 human breast epithelial cells resulted in cells with a tumorigenic phenotype.

P185^{neu}, like many other proto-oncogenes/oncogenes, is a potent PTK. During the past several years, considerable attention has been focused on *neu* and PTKs in human breast tumorigenesis (2). Clearly, the phosphorylation of tyrosine is an important regulatory event in cell proliferation and tumorigenesis. In contrast, virtually nothing is known about the expression of cellular PTPases in these processes. Under normal conditions, a dynamic equilibrium most assuredly exists between cellular PTKs and PTPases in the control of the phosphotyrosine state of the cell. The studies described in

this communication are the first to document the elevated expression of specific cellular PTPases in human breast epithelial cells transformed with an oncogene PTK. In each of the independently derived *neu* transformed human breast epithelial cell lines (18-Rn1, 18-Hn1, 18-Rn2), a substantial increase in the expression of LAR and PTP1B PTPases was observed, as measured by mRNA (RT-PCR, Northern blot) and by protein (immunofluorescence) levels. TC-PTP expression was slightly elevated while expression of CD45 was negative in both the parental cells (184B5) and the *neu* transformed cells. The cells which had the greatest expression of $P185^{neu}$ (18-Hn1 cells) had the greatest increase in LAR and PTP1B expression, as assessed by immunofluorescent analysis.

It is conceivable that the observed correlation between elevated *neu* expression and elevated LAR and PTP1B expression is that, after introduction of *neu*, only those cells which constitutively expressed higher levels of PTPases survived. The following evidence argues against this possibility. After *neu* introduction, 18-Rn1 and 18-Rn2 were isolated as pools of many colonies that survived selection suggesting a high rate of successful transduction of the clonal parental cells (184B5) which initially contained very low PTPase levels. A consistent elevation of LAR and PTP1B was observed in both the polyclonal 18-Rn1 and 18-Rn2 cells and the 18-Hn1 clonal cell line. These results suggest a compensatory elevation of specific PTPases in response to *neu* introduction rather than a selection/survival phenomenon.

To further determine whether the expression of LAR and PTP1B correlated with *neu* expression, we compared rat mammary carcinomas induced via two different agents, i.e. the JR-*neu* retrovirus vector and the carcinogen, DMBA. The *neu*-induced carcinomas exhibited a high expression of *neu* compared to DMBA-induced carcinomas.

Most significant was the striking increase in expression of LAR in the *neu*-induced rat mammary carcinomas. Little or no expression of LAR was observed in the DMBA-induced rat mammary carcinomas. PTP1B expression was substantial and comparable in both types of mammary carcinomas. The elevation of LAR in the *neu*-induced rat mammary carcinomas supports our observation in the *neu*-transformed human breast epithelial cells. Furthermore, those rat mammary carcinomas that had the highest expression of *neu* also had the highest expression of LAR. Thus, in two different models of breast carcinoma (*neu*-induced rat mammary carcinoma cells and *neu*-transformed human breast epithelial cells) increased *neu* expression resulted in a substantial increase in LAR expression.

While our studies are the first to document an increase in PTPase expression after *neu* neoplastic transformation of human breast and rat mammary epithelial cells, a recent report by Dixon and coworkers (18) described a similar phenomenon in a fibroblast model. In their study, expression of PTP1 (a rat PTPase with 97% homology to human PTP1B), was increased 30-50% in NIH 3T3 cells as a result of v-*src* transfection (an oncogene of the PTK family).

It is probable that the elevated expression of specific PTPases in the *neu* transformed human breast epithelial cells and the *neu* transformed rat mammary epithelial cells was the result of a compensatory response leading to acquisition and maintenance of metabolic equilibrium with regard to phosphotyrosine levels. However, the important question is whether or not the elevation in expression of these PTPases, and/or the consequent balance between PTPases and *neu*-PTK, had an influence on the growth and tumorigenicity of these cells. Although it is clear in our studies that high expression of

neu is correlated with tumorigenicity, it is conceivable that the imbalance between *neu*-PTK and PTPase expression could modulate this tumorigenic process. In our study, the most progressively growing human breast carcinoma cells (in athymic nude mice), *i.e.*, 18-Hn1 cells, had the greatest imbalance between $P185^{neu}$ expression and LAR/PTP1B expression when compared to the slower growing 18-Rn1 cells. In addition, the aggressively growing human breast carcinoma cell line SK-BR-3 had high expression of $P185^{neu}$ and low levels of expression of all the PTPases which we examined. Thus, it would appear plausible that endogenous LAR and/or PTP1B PTPase activities could counteract the over-expression of $P185^{neu}$ activity, if such a response could be quantitatively sufficient. Such a response by these PTPases could possibly suppress or even completely block the tumorigenicity of these cells. We are currently developing the methodologies to introduce the genes for specific PTPases (i.e., LAR and PTP1B) into 18-Hn1 and 18-Rn1 cells in an effort to test this hypothesis.

In recent years, the concept of tumor suppressor genes has emerged as a viable concept and has generated, considerable attention and interest. The question that now emerges is whether or not specific cellular PTPases qualify as candidate tumor suppressors. LAR is of particular interest in this regard not only because of its increase in expression in *neu* transformed mammary epithelial cell lines and their derived tumors, as reported in this communication, but because of its unique structure, tissue distribution and chromosome location (13,30). LAR is expressed on epithelial and endothelial cells of many different organ types, including the mammary gland (13,30). In unpublished data from our laboratory, we observed that LAR was expressed not only in the cell lines described in this study, but also in an array of primary normal, immortalized and

carcinomatous human breast epithelial cell lines. In addition, the overall structure of LAR (*i.e.* containing a cell adhesion molecule-like extracellular domain and two cytoplasmic PTPase domains) suggests that LAR may play an important role in the regulation of cell growth via cell-cell contact, in addition to or in concert with its role in the modulation of cellular phosphotyrosine levels. Furthermore, the extracellular region of LAR is structurally similar to the product of the colorectal tumor suppressor gene (DCC); approximately 40% of human breast carcinomas show allelic losses of DCC (32). Recently, Streuli *et al.* (30) have determined that the LAR gene is located on human chromosome 1p32-33, a region that contains candidate breast carcinoma suppressor genes (33). The gene for PTP1B may also qualify as a candidate tumor suppressor gene. Recently, Brown-Shimer *et al.* (17) have demonstrated that PTP1B, when transfected into NIH 3T3 cells, was capable of suppressing transformation by *neu*. In addition, Dixon and colleagues (18) reported that over expression of the PTPase, PTP1, in v-*src* transformed NIH 3T3 cells caused a 75% reduction in the ability of these cells to form colonies in soft agar; such cells reverted to a morphology characteristic of the parental cells.

While we have observed a direct correlation between $p185^{neu}$ expression and tumorigenicity in our *neu* transformed human breast epithelial cell lines, it is important to note that the cell lines which had the highest level of $p185^{neu}$ expression also had the highest expression of LAR and PTP1B. This might suggest that the increase in these PTPases may actually enhance the tumorigenicity of these cells. Indeed such a phenomenon has been recently reported (34). Although we cannot rule out this possibility, the observations involving PTP1B and PTP1 (17,18) noted above strongly

support the hypothesis that LAR/PTP1B are acting to suppress the transformed phenotype. Furthermore, no evidence has been reported to date documenting a stimulatory effect of PTP1B or LAR in cell proliferative processes.

The mechanism by which PTPase expression is increased in cells with increased neu expression is currently unknown. The balance between *neu*-PTK and PTPase activities could be at the transcriptional, translational or post-translational levels. This is no doubt a complicated process, probably involving more than one regulatory system. We were, however, able to rule out the possibility of gene amplification, based on our results of Southern hybridization analysis.

In summary, we have observed that the certain PTPases substantially increase in expression in human breast epithelial cells (LAR and PTP1B) and in rat mammary epithelial cells (LAR) as a result of *neu*-PTK induced neoplastic transformation. While the role of these genes in this tumorigenic process is presently not known, the results of our studies provide evidence that specific PTPases do indeed respond, with increased expression, to a tumorigenic stimulus brought about by an introduced oncogene (*neu*). Such PTPases warrant thorough examination as to their role in this tumorigenic process.

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CHAPTER III

INFLUENCE OF INCREASED EXPRESSION OF LEUKOCYTE COMMON ANTIGEN RELATED (LAR)-PROTEIN TYROSINE PHOSPHATASE ON HUMAN BREAST CARCINOMA TRANSFORMED BY THE *NEU* ONCOGENE

FOOTNOTES

Abbreviations: PTPase, protein tyrosine phosphatase; PTK, protein tyrosine kinase; LAR, leukocyte-common antigen related; BHK, baby hamster kidney; MT, metallothionein; MCS, multiple cloning site.

ABSTRACT

Protein tyrosine phosphorylation/dephosphorylation is a fundamental mechanism controlling cell proliferation, differentiation and neoplastic transformation, which is regulated via the opposing activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases). It has been proposed that the genes encoding certain PTPases could be tumor suppressor genes because of their ability to counteract PTK activity. To directly assess this hypothesis, a full length of cDNA of a transmembrane type PTPase, leukocyte common antigen related PTPase (LAR), was successfully cloned into an inducible expression vector. The expression of LAR was modulated via transcription from a metallothionein (MT) promoter. The LAR containing plasmid was introduced into a *neu* transformed human breast carcinoma cell line, 18-Hn1, that has high PTK activity, This resulted in LAR overexpression in these cells, morphological changes *in vitro* and significant suppression of tumorigenicity when these cells were inoculated into athymic nude mice. These results provide evidence that an elevation in PTPase expression in breast carcinoma cells with high $p185^{neu}$-PTK expression can substantially change the biological behavior of these cells, in particular, a reduction in tumorigenicity. Such results support the hypothesis that certain PTPase genes could play a role as tumor suppressor genes, in particular in these tumor cells that have a high level of $p185^{neu}$-PTK expression.

INTRODUCTION

Tyrosine phosphorylation/dephosphorylation appears to be a key regulatory mechanism in the control of signal transduction, cell proliferation, differentiation and transformation (Ullrich and Schlessinger, 1990; Fischer et al, 1991; Saito and Streuli, 1991 and Brautigan, 1992). The net cellular level of tyrosine phosphorylation is maintained dynamically by the opposing action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases). While the involvement of PTKs in these cellular processes has been extensively examined, an examination of the role of PTPases in these processes has only just begun.

The *neu* oncogene (c-*erb*B-2 or HER-2), encodes a 185 kDa transmembrane glycoprotein, $P185^{neu}$, with PTK activity. The human *neu* oncogene has been found to be amplified and overexpressed in approximately 30% of primary human breast carcinomas (Kraus et al, 1987; Slamon et al, 1987; Van de Vijver et al, 1987) and to be correlated with poor prognosis (Slamon et al, 1987, 1989, Borg et al, 1991). Experimentally, it has been demonstrated that the constitutive activation of *neu* PTK activity resulting in hyperphosphorylation of cellular substrates, is important, perhaps essential, for transforming activity (Van de Vijver et al, 1991, Pierce et al, 1991, Zhai et al, 1993). These findings suggest that hyperphosphorylation resulting from *neu* activation could be a crucial factor in the development of human breast carcinomas.

PTPases, on the other hand, comprise a diverse family of enzymes that exist as cytosolic, nonreceptor and receptor type transmembrane forms. Since many oncogenes encode proteins which have an intrinsic PTK activity essential for transformation, and since PTPases have the potential to counteract the activity of PTKs, it has been proposed

that PTPase genes could function as tumor suppressor genes (Fischer et al, 1991; Stanbridge, 1990). Conceptually, the lack of dephosphorylation resulting from a loss of PTPase activity could be oncogenic. In contrast, overexpression of a PTPase could reverse neoplastic transformation by oncogenic PTKs. Indeed, Laforgia *et al.* have reported that the loss of one allele of the human RPTPγ gene (which encodes a receptor type PTPase) occurred in approximately one-half of the tumor samples examined (Laforgia et al, 1991). Evidence in further support of this hypothesis includes the following observations: 1) when PTP1B was introduced into NIH3T3 cells, the overexpression of this cytosolic PTPase suppressed transformation by *neu* (Brown-Shimer et al, 1992) and v-*src* (Woodford-thomas et al, 1992) oncogenes. Similarly, overexpression of an unidentified cytosolic PTPase in v-*erb*B infected NIH3T3 cells suppressed neoplastic transformation (Ramponi et al, 1992). 2) The overexpression of the carboxyl-terminal truncated TC-PTP (cytosolic PTPase) in BHK cells resulted in approximately 50% reduction in growth rate. In addition, morphological changes and multinucleation resulting from cytokinetic failure and asynchronous nuclear division were observed in these cells (Cool et al, 1990, 1992). 3) The expression of PTP1B was significantly increased in Swiss 3T3 cells as well as in normal fibroblast cells in response to density-dependent contact inhibition (Pallen et al, 1991); these data were consistent with similar observations using monkey kidney epithelial CV-1 cells (Brautigan et al, 1991); 4) Treatment with the PTPase inhibitor, vanadate, caused a phenotypic transformation of fibroblasts. It was suggested that this transformation was due to increasing the phosphotyrosine content within these cells, which led to a decreased density-dependent growth inhibition (Klarlund et al, 1985). Collectively, these findings

indicate that PTPases have anti-oncogenic potential in which inactivation of such genes by mutation, deletion, and/or suppression by potent PTPase inhibitors, could lead to tumorigenesis.

Numerous studies have been performed to address the role of $P185^{neu}$-PTK in human breast cancerigenesis. In contrast, nothing is known as to whether or not PTPases can act as inhibitors of this tumorigenic process. We have addressed this issue using two different approaches. One approach was aimed at studying whether or not the introduction of an activated *neu* oncogene into an immortalized human breast epithelial cell line, which caused neoplastic transformation of these cells, could result in alterations of PTPase expression. In the second approach, we sought to determine whether or not introduction of a PTPase gene into the *neu* transformed human breast carcinoma cell line, would be able to reverse neoplastic transformation by partially or completely suppressing the tumorigenicity of these cells. The first approach led to the finding of a significant elevation in the expression of the PTPases LAR (a receptor PTPase) and PTP1B in three independently *neu* transformed human breast carcinoma cell lines in response to *neu* introduction (Zhai et al, 1993). The second approach is the subject of this communication. Among the PTPase family, LAR is of particular interest to us, not only because it is sharply elevated in *neu*-PTK transformed human breast carcinoma cells (Zhai et al, 1993), but also because of its unique structure, tissue distribution and chromosome location (Streuli et al, 1988, 1989, 1992). To test the anti-oncogenic potential of LAR PTPase, we constructed a plasmid containing the human LAR cDNA, whose expression is modulated from an inducible metallothionein (MT) promoter. The introduction of this plasmid into a *neu* transformed human breast carcinoma cell line and

the effects of overexpression of LAR on the tumorigenic activity of this cell line is the subject of this communication.

MATERIALS AND METHODS

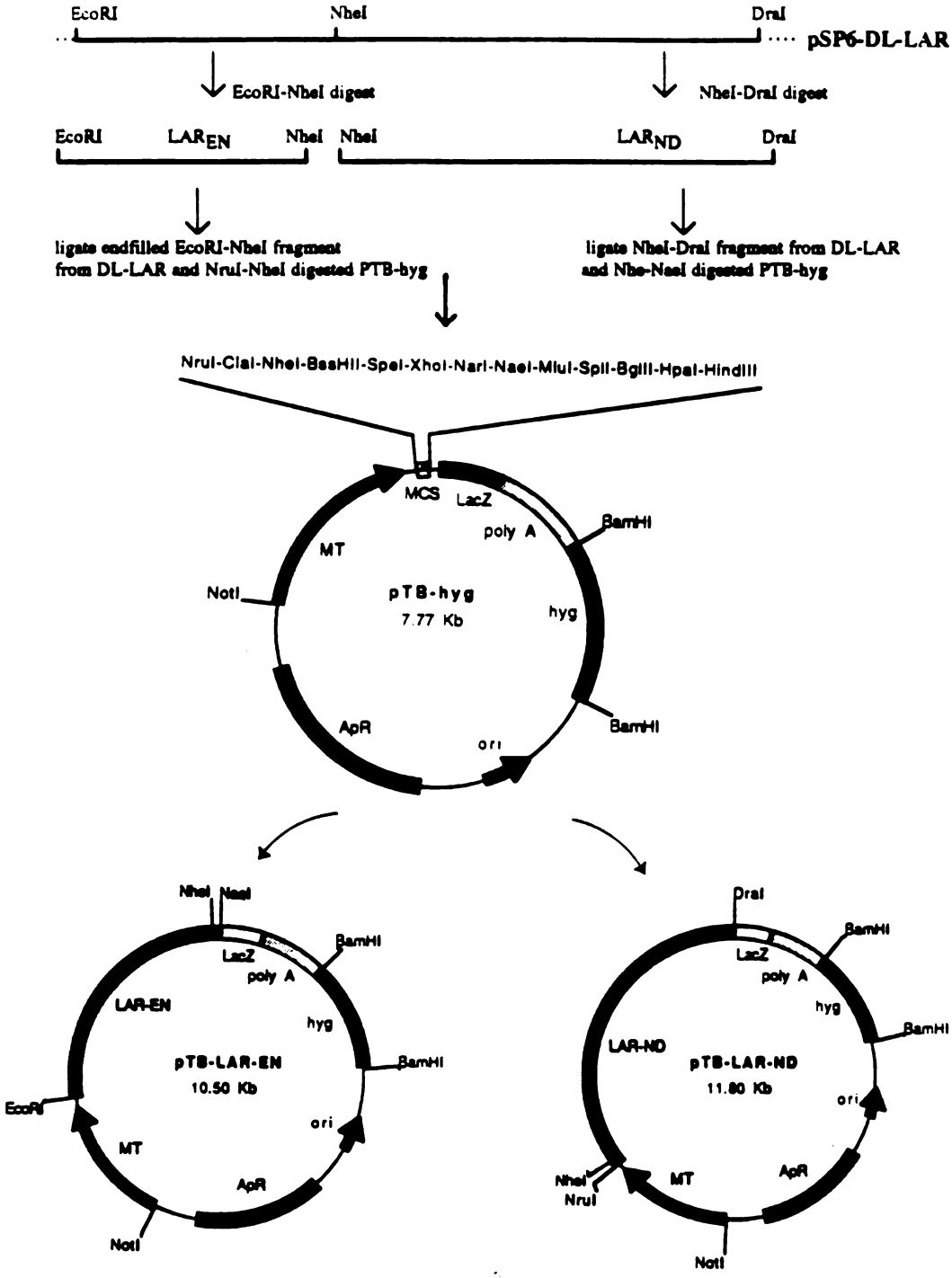
Cell Culture. *Neu* transformed human breast carcinoma cell line 18-Hn1 was the gift of Dr. J. Pierce (National Cancer Institute, Bethesda, MD). 18-Hn1 and LAR-transfected 18-Hn1 clones (18-Hn1-LAR) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (GIBCO, Grand Island, NY) and 10^{-3} dexamethasone (Sigma Chemical Co., St. Louis, MO).

Construction of The Vector Expressing Human LAR cDNA. The plasmid pSP6.DL-LAR, which contains the human LAR cDNA was a generous gift of Dr. M. Streuli (Dana-Farber Cancer Institute, Boston, MA). A three-step procedure was used for the construction of pTB-LAR_{ED} (from EcoRI to DraI). First, a 2.6 kb end-filled EcoRI+NheI fragment PTB-LAR_{EN} digested from PSP6.DL.LAR was ligated to NruI+NheI digested PTB-hyg (a gift from Dr. J. McCormick, Michigan State University, E. Lansing, MI), which contained an inducible MT promoter, a multiple cloning site (MCS) and a hygromycin resistance gene as a selection marker (Wang et al, 1992). This ligation led to the generation of a 10.5 kb LacZ-α-based intermediate plasmid PTB-LAR_{EN}. Secondly, a NheI+DraI fragment (4.1 kb) from PSP6.DL.LAR was cloned into pTB-hyg by NheI+NaeI digestion and ligation. This produced another intermediate plasmid pTB-LAR_{ND} (11.8 kb). Thirdly, the plasmid PTB-LAR_{ED} was constructed by cloning a 4.7 kb NotI+NheI fragment from pTB-LAR_{EN} into pTB-LAR_{ND} by NotI+NheI digestion and ligation (Fig. 1). After bacterial transformation, clones obtained from each step were screened on XGal plates by LacZ-α-

Figure 1. Strategy for a construction of a full length LAR cDNA expression vector utilizing a MT promoter and a hygromycin selection marker. The final vector was constructed in three steps (see Materials and Methods). The resulting plasmid, pTB-LAR_{ED} (14.5 kb) contains an inducible metallothionein promoter (MT), a LacZ fragment, the LAR coding sequence (6.7 kb from EcoRI to DraI sites), a SV-40 poly-A and a hygromycin-resistance gene as a selection marker.

STRATEGY FOR THE CONSTRUCTION OF THE LAR EXPRESSION VECTOR.

Subclone two fragments of LAR into pTB-*hyg*.

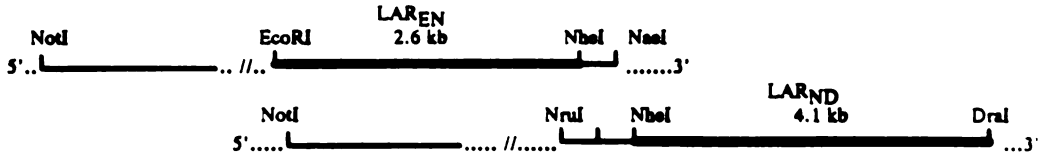


Isolate and Verify Two Intermediate Plasmid Products

- bacterial transformations using JM109 cells
- screen colonies on X-Gal plates (isolate white colonies)
- verification of insertion and orientation by restriction digestions

pTB -LAR$_{EN}$ (10.5kb)
(1 of 26 colonies)

pTB -LAR$_{ND}$ (11.8kb)
(10 of 10 colonies)



Ligate the two LAR fragments.

pTB-LAR$_{EN}$ ↓

pTB-LAR$_{ND}$ ↓

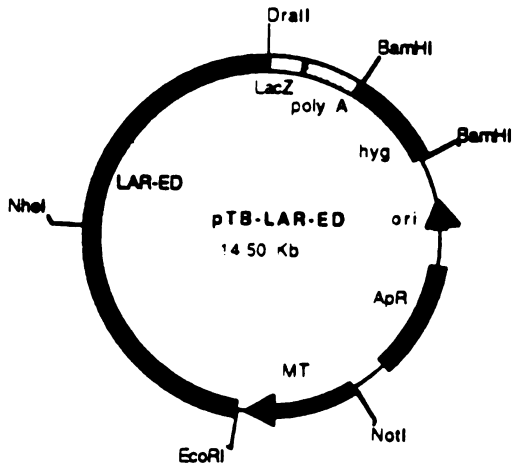
NotI-NheI digest, electroelution

NotI-NheI fragment form pTB-LAR$_{EN}$, 4.7kb
(contains EcoRI-NheI LAR fragment)

NotI-NheI digested pTB-LAR$_{ND}$, 9.8kb
(contains NheI-DraI LAR fragment)

LIGATION

↓



↓ bacterial transformation, colony screening, restriction digestion

↓ DNA transfection

LAR expression, cells at complementation using *E. coli* host strain JM109 containing an amber suppressor gene. The orientation of the insertions were analyzed by restriction digestion.

DNA Transfection and Selection of Clones. 18-Hn1 cells were transfected with pTB-LAR_{ED} or its control plasmid pTB-hyg DNA using lipofectin (GIBCO) as described by the supplier with minor modification. Briefly, 5 x 10^5 cells were seeded in a P100 tissue culture dish in serum containing growth medium. After 24 hours, 4 ml of lipofectin reagent-DNA complex (containing 30 μg of plasmid DNA and 30 μl of lipofectin reagent) were added to each dish and incubated overnight at 37°C. The DNA containing medium was replaced with serum containing growth medium followed by incubation for another 48 hours to allow the expression of resistance gene. Hygromycin (75 μg/ml) was then added for selection of stable colonies.

Effects of LAR Overexpression on Cell Morphology and Growth. To induce expression of the MT-LAR gene, $ZnSO_4$ was added to the culture medium achieving final concentrations ranging from 100 to 400 μM. The effect of LAR overexpression on 18-Hn1 cell growth rate *in vitro* was measured by plating 2 x 10^4 cells on 60 mm dishes on day 0, $ZnSO_4$ was added on day 1 and the cells were counted on day 1, 4 and 7. The effect of LAR overexpression on morphological changes was assessed and photographed on day 5. The effect of $ZnSO_4$ concentration on cell density dependent inhibition was measured by plating 2 x 10^4 cells on day 0, followed by the addition of $ZnSO_4$ on day 1 and cell counting on day 7. To determine the effect of different cell densities on three different densities (5 x 10^5, 1 x 10^6, 1 x 10^7) were added to 100 mm dishes followed by

daily treatment with 200 μM $ZnSO_4$. The cells were then harvested and subjected to immunoblot analysis using anti-LAR monoclonal antibody.

Immunoprecipitation and Western Blot Analysis. The monoclonal anti-human LAR antibody 11.1A was a generous gift from Dr. M. Streuli. Anti-human c-*erb*B-2 monoclonal antibody was obtained from Oncogene Science, Inc. (Uniondale, NY). Monoclonal anti-phosphotyrosine and anti-human phospholipase C-γ1 (PLC-γ1) antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). LAR, $p185^{neu}$ protein expression and tyrosine phosphorylation of cellular proteins in 18-Hn1 cells, control plasmid containing 18-Hn1 cells, and pTB-LAR transfected 18-Hn1 cells were assessed by immunoblot analysis as follows. The cells were washed with ice-cold PBS and scraped into lysis buffer [20 Mm Tris pH 8.0/137 Mm NaCl/10% glycerol/1% NP-40/2 mM phenyl-methylsulfonal fluoride (PMSF)/0.15 U/ml aprotinin/5 Mm EDTA] with or without 1 mM PTPase inhibitor sodium orthovanadate (Sigma) at 4°C for 30 min. After centrifugation (12,000 rpm, 20 min), lysates were either immediately boiled for 3 min in sample buffer for immunoblotting or stored at -80°C. The protein concentration in the lysates was determined by the Bio-rad protein assay. Equivalent amounts of protein from each cell line were electrophoretically separated by 8% SDS-PAGE, and transferred onto Immobilon-P transfer membranes (Millipore Corp., Bedford, MA). Immunoblots were probed with the appropriate antibody, washed five times with TBST (20 mM Tris, 150 mM NaCl, 0.01% Tween 20) and then incubated with a secondary antibody (goat anti-mouse IgG-horseradish peroxidase conjugated, Boehringer Mannheim Biochemicals, Indianapolis, IN). The blots were washed five times and were visualized using the ECL chemiluminescence system (Amersham Corp., Arlington

Heights, IL). The procedure for stripping and reprobing of membrane was performed as described by supplier (Amersham).

Tumorigenicity Assay. pTB-LAR and control plasmid transfected 18-Hn1 cells (5×10^6 cells per cell line) were inoculated s.c. into 6 week old female athymic nude mice (Harlan Sprague-Dawley, Indianapolis, IN). To determine the effect of zinc on expression of the MT-LAR gene, half of the cells were pretreated with 200 μM $ZnSO_4$ for 24 hours before injection into athymic nude mice. The diet of the athymic nude mice bearing zinc treated cells contained an additional 200 ppt $ZnSO_4$. The mice were fed ad libitum a standard rat/mouse chow (Wayne Lab Blox, Allied Mills, Chicago, IL) and were maintained in our pathogen-free barrier facility (germ free laminar airflow); drinking water, food, bedding and cages were sterilized prior to use. Tumor diameters were measured at weekly intervals and tumor weights were obtained when the experiment was terminated 4-5 weeks after cell inoculation. Statistical analysis of these data were determined by the unpair student's T-test and by one-way analysis of variance and the Neuman-Keuls multiple comparison test.

RESULTS

Vector Construction and Verification. A three-step construction protocol resulted in a plasmid, PTB-LAR_{ED} (EcoRI to DraI), containing an inducible MT promoter, a LacZ fragment with a MCS, the full length LAR coding sequence (6.7 kb from EcoRI to DraI sites), an SV-40 polyadenylation site, and a hygromycin resistance gene (hyg). The LAR cDNA was cloned into the LacZ-based MCS containing an amber stop codon which resulted in white colonies on X-Gal plates after successful plasmid insertion. The two intermediate plasmids (pTB-LAR_{EN} and pTB-LAR_{ND}) and the final plasmid (pTB-LAR_{ED}, designated pTB-LAR hereafter) were analyzed using multiple restriction enzyme digestions to ensure the correct size and orientation of the inserts (Table 1 and Figure 2).

Transfection and Selection of 18-Hn1 Colonies With Expression of MT-LAR_{ED}. To assess the effects of overexpression of human LAR PTPase on oncogenic or transforming properties of $p185^{neu}$, 18-Hn1 cells were transfected with the inducible eukaryotic expression vector (pTB-LAR) containing an intact human LAR cDNA sequence or with the expression vector alone (pTB-hyg) as a control. Three days after lipofectin-mediated transfection, hygromycin was added to the culture medium (75 μg/ml) for selection. Two to three weeks later, a number of stable, independent cell lines were isolated and expanded for further analysis. The stable expression of the hygromycin drug-resistance marker was confirmed since all of the lines were resistant to 75 μg/ml hygromycin, a level which was toxic to the parental cells. The hygromycin resistant colonies were designated 18-Hn1-LAR_1, 18-Hn1-LAR_2, *etc.*

Figure 2. Restriction enzyme digestion of the intermediate and full length LAR expression vectors. The orientation and size of the LAR constructs were verified by digestion with BamHI (panel A), NsiI+XhoI (panel B), or NotI+NheI (panel C). The digestion products were separated by 1% agarose gel and visualized by ethidium bromide staining. Lane 1, Lambda DNA-HindIII digest; lane 2, pTB-hyg; lane 3, pTB-LAR_{EN}; lane 4, pTB-LAR_{ND}; lane 5, pTB-LAR_{ED}; lane 6, Lambda DNA-BstEII digest. The size of the digest products are summarized in Table I.

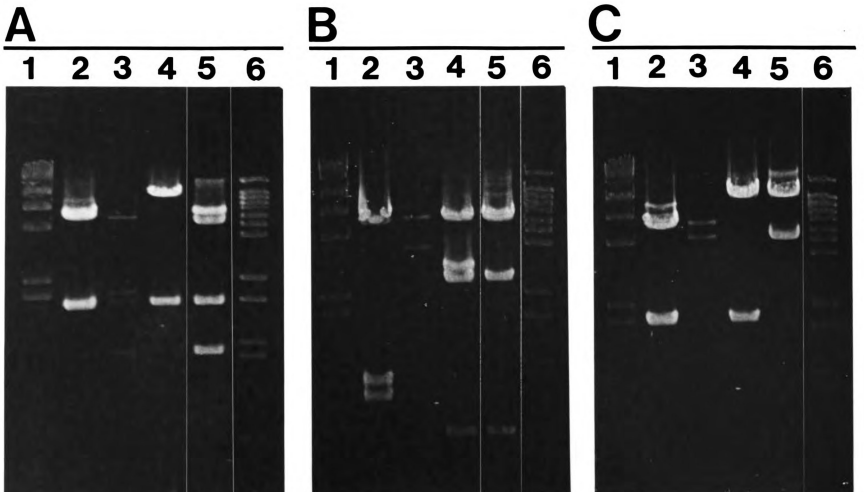

A
1 2 3 4 5 6
B
1 2 3 4 5 6
C
1 2 3 4 5 6

Table 1. Multiple restriction enzyme digestion of plasmid DNA.

Restriction enzyme	Length of digestion products (kb)			
	pTB-hyg	pTB-LAR$_{EN}$	pTB-LAR$_{ND}$	pTB-LAR$_{ED}$
BamHI	5.85	5.30	9.90	5.90
	1.92	1.92	1.92	5.30
		2.00		1.92
		1.31		1.31
NsiI+XhoI	5.80	5.80	5.80	5.80
	1.15	3.55	2.70	5.35
	0.86	1.15	2.60	2.60
			0.75	0.75
NotI+NheI	5.8	5.8	9.8	9.8
	2.0	4.7	2.0	4.7
Total length	7.8	10.5	11.8	14.5

To verify expression of LAR protein, whole cell lysates from pTB-LAR and control plasmid transfected and wild type 18-Hn1 cells were prepared and subjected to immunoblotting utilizing monoclonal anti-human LAR (mAb 11.1A). Western blot analysis of uninduced 18-Hn1-LAR cells indicated that LAR expression was higher in some of the 18-Hn-LAR cell lines (Fig. 3A, lanes 4 and 5) compared to the parental cells and cells containing the control plasmid (Fig.3A, lanes 1, 2, and 3). Further elevated levels of LAR protein were detectable in pTB-LAR transfected cell lines after daily treatment with $ZnSO_4$ (200-400 μM) (Fig 3A and 3B, lanes 4-7). The presence of $ZnSO_4$ had no effect on LAR expression in 18-Hn1 cells and cells containing the control plasmid pTB-hyg (Fig. 3A and 3B, Lane 1-3). Stripping and reprobing the same membrane with anti-*erb*B2 monoclonal antibody showed that the $p185^{neu}$ expression was comparable among 18-Hn1, control plasmid transfected 18-Hn1 and 18-Hn1-LAR cells. Thus, there was no effect of overexpression of LAR on $p185^{neu}$ expression in 18-Hn1-LAR cells (Fig. 3C and 3D).

Effects of Overexpression of LAR on Morphology and *In Vitro* Growth of 18-Hn1-LAR Cells. Alterations in morphological appearance were observed in several independent 18-Hn1-LAR cell lines which were distinct from that of wild type 18-Hn1 and 18-Hn1 cells containing the pTB-hyg control vector alone. The most obvious effect was observed in subconfluent cultures, with or without $ZnSO_4$ treatment. The 18-Hn1-LAR cells were more flattened, had wide variation in size and shape and had increased amounts of granules or vesicles in their cytoplasm (Fig. 4B-D). The vesicles were predominantly located close to the nucleus (Fig. 4C, 4D). In addition, there was an increased proportion of large bi- and multi-nucleate cells (Fig. 4D).

Figure 3. Comparison of expression of LAR and p185^{neu} in 18-Hn1 cells and 18-Hn1-LAR cells (pTB-LAR transfected). Cells were harvested 48 hours after treatment with 200 μM $ZnSO_4$ (panel B and D) or without $ZnSO_4$ (panel A and C). Total cell lysates (500 μg) from each cell line were separated by SDS-PAGE and subjected to Western blot analysis using anti-LAR mAb (panel A and B). The blot was stripped and reprobed with anti-p185^{neu} mAb (panel C and D). Lane 1, 18-Hn1 cells; lanes 2 and 3, 18-Hn1-C, cells transfected with pTB-hyg; lane 4, 18-Hn1-LAR_4; lane 5, 18-Hn1-LAR_5; lane 6, 18-Hn1-LAR_7; lane 7, 18-Hn1-LAR_{10}.

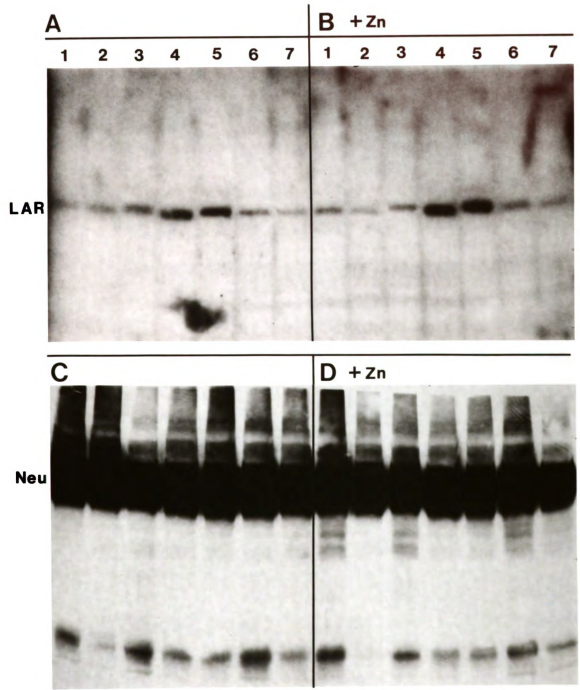
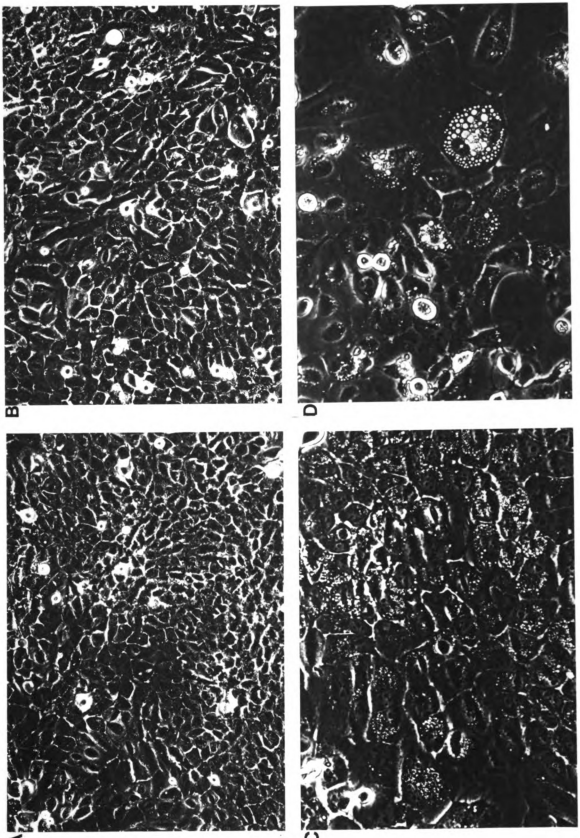

A
B +Zn
1 2 3 4 5 6 7
1 2 3 4 5 6 7
LAR
C
D +Zn
Neu

Figure 4. Morphological appearance of 18-Hn1 cells and 18-Hn1-LAR maintained *in vitro*. All cells were grown in medium containing 200 μM $ZnSO_4$. 18-Hn1 cells, panel A (37.5 fold enlargement); 18-Hn1-LAR cells, panel B, C and D at different magnifications: 37.5, 70 and 70 respectively.


A
B
C
D

The effect of overexpression of LAR on 18-Hn1 cell growth as a function of $ZnSO_4$ concentration is shown in Fig. 5. The doubling time of 18-Hn1-LAR cells, increased only 1-2 hours compared to control plasmid containing 18-Hn1 cells, regardless of the absence or presence of different concentrations of $ZnSO_4$ (Fig. 5). Although 18-Hn1-LAR cells grew more slowly as a result of increasing $ZnSO_4$ concentration, increasing the zinc concentration also slowed the growth of 18-Hn1 cells with the control plasmid. This suggests that the reduction of growth rate of 18-Hn1-LAR cells with increasing $ZnSO_4$ concentration was the result of $ZnSO_4$, rather than an effect of LAR overexpression.

The effect of density-dependent cell contact inhibition on LAR expression in 18-Hn1 and 18-Hn1-LAR cells was also examined (Fig. 6). The expression of LAR was further elevated as a result of increasing cell density in both 18-Hn1 and 18-Hn1-LAR cells.

Effect of LAR Overexpression on Tyrosine Phosphorylation in 18-Hn1 and 18-Hn1-LAR Cells. The change in the phosphotyrosine content of cellular proteins as a consequence of LAR introduction in 18-Hn1 cells was analyzed using anti-phosphotyrosine mAb (α-PY). In 18-Hn1-LAR cells, there was a lower level of tyrosine phosphorylation in several protein bands, particularly those of 71, 85 and 91 kDa, but not that of the 60, and 185 kDa proteins (Fig. 7). These results suggest that overexpression of LAR in 18-Hn1-LAR cells resulted in a reduction of phosphotyrosine content of several cellular proteins in these cells.

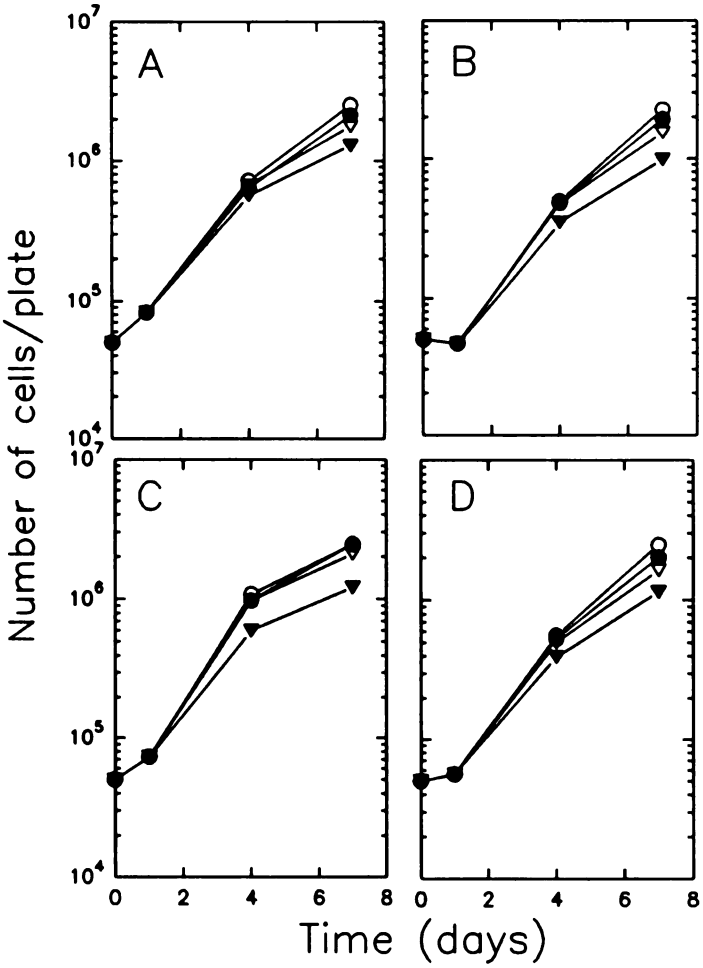


Figure 5. Effect of $ZnSO_4$ supplementation on the growth rate of 18-Hn1-LAR cells *in vitro*. Cells were plated at equal numbers (2 X 10^4) and $ZnSO_4$ was added at the indicated concentrations. Cells were harvested after 1, 4 and 7 days of incubation and the counts for three plates were averaged. $ZnSO_4$ concentrations are as follows; none (-○-), 100 μM (-●-), 200 μM (-▽-) and 400 μM (-▼-). Panel A, 18-Hn1 cells containing pTB-hyg; panel B, 18-Hn1-LAR_4; panel C, 18-Hn1-LAR_5; panel D, 18-Hn1-LAR_7.

Figure 6. Western blot analysis of cell density dependent LAR expression in 18-Hn1 cells and in 18-Hn1-LAR_5 cells. The cells were cultured under different cell densities as indicated and after lysis equivalent amounts of protein (100μg per lane) were analyzed by SDS-PAGE. Immunoblot analysis was performed using anti-LAR mAb. The position of LAR is indicated.

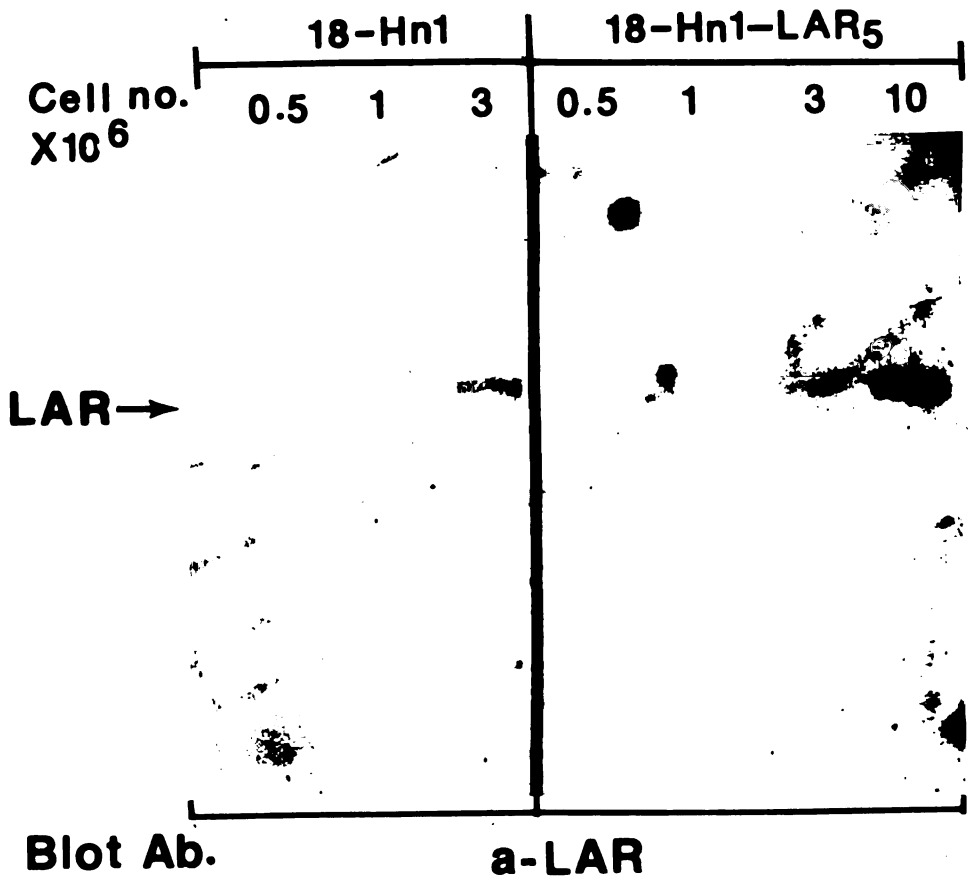
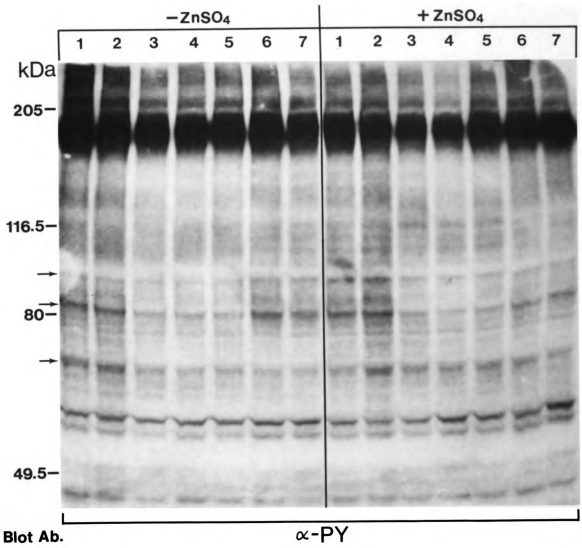

18-Hn1
18-Hn1-LAR5
Cell no.
X10^6
0.5
1
3
0.5
1
3
10
LAR→
Blot Ab.
a-LAR

Figure 7. Western blot analysis of phosphotyrosine-containing proteins of 18-Hn1, control plasmid containing 18-Hn1 cells and 18-Hn1-LAR cells with or without $ZnSO_4$ treatment. The cells were lysed in the presence of 1mM sodium orthovanadate (see Materials and Methods). Equivalent amounts of protein (150 μg per lane) from total cell lysate were analyzed by 8% SDS-PAGE and immunoblotting was performed with anti-phosphotyrosine antibody (α-PY). The identification of cellular proteins whose tyrosine phosphorylation level appears reduced is shown (arrows). Lane 1, 18-Hn1 cells; lane 2, 18-Hn1-C, cells with control plasmid; lane 3, 18-Hn1-LAR_4; lane 4, 18-Hn1-LAR_5; lane 5, 18-Hn1-LAR_7; lane 6, 18-Hn1-LAR_{10}; lane 7, 18-Hn1-LAR_{13}.


−ZnSO4
+ZnSO4
1 2 3 4 5 6 7
1 2 3 4 5 6 7
kDa
205
116.5
80
49.5
Blot Ab.
α-PY

Effect of LAR Introduction on Tumorigenicity of 18-Hn1 Cells in Athymic Nude Mice. The ability of five 18-Hn1-LAR cell lines (#4, 5, 7, 10 and 13) to form tumors in athymic nude mice was compared to 18-Hn1 cells transfected with the control plasmid alone. This study was conducted both without and with $ZnSO_4$ addition to the diet (Table 2). Each cell line examined readily formed palpable tumors after s.c. inoculation into athymic nude mice regardless of the presence or absence of $ZnSO_4$ treatment. Mean tumor weights and volumes were numerically smaller in each of 18-Hn1-LAR cell lines (#4, 5, 7, 10 and 13), compared to that observed in 18-Hn1 cells containing pTB-hyg plasmid alone. Significant ($P<0.05$) mean differences were observed when comparing controls (18-Hn1-C) to cell lines 18-Hn1-LAR #4, 5, 7 and 10 in both conditions without and with ZnSO4 supplementation.

Table 2. Tumorigenicity of 18-Hn1 Cells and 18-Hn1-LAR Cells in Athymic Nude Mice

Cell line[a]	Regular Diet			Diet With $ZnSO_4$		
	Number of tumors	Tumor weight g ± SEM	Tumor volume cm^3 ± SEM	Number of tumors	Tumor weight g ± SEM	Tumor volume cm^3 ± SEM
18-Hn1-C	20	0.570 ± .07[b]	2.177 ± .47[b]	22	0.510 ± .07[b]	1.427 ± .21[b]
18-Hn1-LAR_4	19	0.130 ± .04[c]	0.307 ± .08[c]	20	0.320 ± .04[c]	0.608 ± .09
18-Hn1-LAR_5	18	0.170 ± .05[c]	0.302 ± .08[c]	18	0.240 ± .05[c]	0.397 ± .11[c]
18-Hn1-LAR_7	10	0.320 ± .06[c]	0.600 ± .15[c]	15	0.160 ± .04[c]	0.456 ± .11[c]
18-Hn1-LAR_{10}	17	0.240 ± .06[c]	0.408 ± .08[c]	15	0.190 ± .05[c]	0.349 ± .08[c]
18-Hn1-LAR_{13}	14	0.360 ± .10	0.743 ± .19[c]	14	0.280 ± .07[c]	0.652 ± .19

[a] 18-hn1 and 18-Hn1-LAR cells are inoculated s.c. into female athymic nude mice. Animals were sacrificed 4-5 weeks after cell inoculation.
[b/c] $p<0.05$

DISCUSSION

The results of this study provide evidence for: (1) the construction of an effective LAR cDNA expression vector with an inducible promoter; (2) the effective introduction of the LAR gene into *neu* transformed human breast carcinoma cells (18-Hn1 cells) resulting in cell lines with a elevation in LAR expression; (3) a change in the morphological appearance (increased cellular size and cytoplasmic granularity) of the LAR transfected cells; and (4) a significant reduction in tumorigenicity of the LAR cells when inoculated into athymic nude mice.

The human LAR cDNA expression vector (pTB-LAR) was constructed to incorporate both an inducible MT promoter region and a hygromycin resistance gene as a selection marker. The advantages of this inducible expression vector are as follows. First, the hygromycin resistance gene of PTB-LAR allowed the transfection of cells which had previously been transfected by a construct containing the *neu* oncogene and the neomycin resistance gene. Second, the expression level of LAR was at least partly under the control of an inducible MT promoter. This potentially facilitates a more precise control of the expression of LAR. Third, introduction of the human LAR cDNA into a LacZ based MCS allowed isolation of those colonies expressing LAR by choosing white colonies on X-Gal plates (using *E. coli* host JM109 based on α-complementation). Thus, the pTB-LAR expression vector facilitated the study of the interactions between LAR-PTPase and $P185^{neu}$-PTK activity in human breast cancer cell lines.

Immunoblot analysis showed that the expression of LAR was increased in all of the 18-Hn1-LAR cell lines examined, compared to 18-Hn1 cells containing the control plasmid. Further elevation of LAR expression is detectable in 18-Hn1-LAR cell lines

after ZnSO4 treatment, while the presence of zinc had no effect on LAR expression in 18-Hn1 cells. These data provide evidence that the expression levels of LAR in 18-Hn1-LAR cell lines are inducible.

Overexpression of LAR in 18-Hn1-LAR cells resulted in morphological changes when compared to either 18-Hn1 cells or 18-Hn1 cells containing the pTB-hyg vector only. LAR overexpression increased the number of enlarged cells and increased the proportion of bi- and multi-nucleate cells. In addition, increased amounts of granules (or vesicles) were evident in the LAR transfected cells. The granules were located predominantly in the perinuclear cytoplasm. These observations are consistent with those found in other laboratories in which NIH3T3 and BHK cells showed similar morphological changes after transfection with the genes encoding the PTP1B PTPase (Woodford et al, 1992) or truncated TC-PTP PTPase (Cool et al, 1990, 1992). Overexpression of the carboxyl-terminal truncated TC-PTP in BHK cells resulted in cytokinetic failure and asynchronous nuclear division, leading to a multinucleate morphology (Cool et al, 1990, 1992). This suggests that the small proportion of bi- and multi-nucleate cells in the 18-Hn1-LAR clones may also have arisen from a failure of cell division as a result of overexpression of LAR. That the overexpression of LAR had no or little effect on cell growth rate *in vitro,* however, argues against a major defect in cell division. Alternatively, it is possible that the multi-nucleated cells were the result of differentiation of cells in response to overexpression of LAR, a phenomenon that had been described in keratinocytes (Zhao et al, 1992), neuronal cells (Aparicio et al, 1992; den Hertog et al, 1993), and more frequently, in hematopoietic cells (Butler et al, 1990; Buzzi et al, 1992; Cohen et al, 1992; and Zafriri et al, 1993). These cells undergo

differentiation associated with increasing PTPase activities after treatment with maturational agents, such as 12-O-tetradecanoylphorbol-13-acetate (PMA) and dimethyl sulfoxide (DMSO) (Cohen et al, 1992; Butler et al, 1990, Buzzi et al, 1992), IL-6 (Zafriri et al, 1993), or after transfection of CD45 PTPase (Buzzi et al, 1992). In our studies, it will be of importance to further characterize multinucleate 18-Hn-LAR cells; such cells may represent a differentiated phenotype of cells with elevated expression of PTPases.

It is necessary to consider the possibility that the morphological changes associated with the 18-Hn1-LAR cell lines may have been due to the effects of zinc cytotoxicity rather than induction of expression of an exogenous LAR gene. Among other properties, it is known that the divalent heavy metal zinc is a PTPase inhibitor (Wang et al, 1992; Tahiri-Jouti et al, 1992; Pot et al, 1991 and Itoh et al, 1992). However, it has been demonstrated that both human and rat LAR PTPase activities were not inhibited by zinc at concentrations up to 1,000 μM *in vitro*, a concentration much higher than those used in our studies (Pot et al., 1991; Itoh et al, 1992). These morphological alterations were also observed in 18-Hn1-LAR cells that were not treated with zinc, albeit to lesser degree. Thus, this alternative explanation does not seem likely.

Overexpression of LAR in 18-Hn1 cells resulted in significantly suppressed tumorigenicity of such cells when inoculated into athymic nude mice. This suppression occurred in spite of the fact that LAR overexpression did not affect cell growth rate *in vitro*. This apparent inconsistency may be interpreted as due to an increased frequency of cell-cell contact inhibition *in vivo*, rather than a direct inhibition of cell division (proliferation). Evidence to further support this explanation include the following. First,

increased LAR expression was observed as a consequence of increased cell density in both 18-Hn1 and 18-Hn1-LAR cells suggesting that LAR expression *in vitro* was regulated in part by cell density. Second, LAR may play an important role in the regulation of cell growth via cell-cell contact due to the cell adhesion molecule (CAM) like structure on its extracellular domain (Streuli et al., 1988; 1990; 1992). This effect could occur in addition to, or in concert with, the role of LAR in the modulation of cellular phosphotyrosine. Third, Pallen *et al.* (1991) have demonstrated that membrane PTPase activity in Swiss 3T3 and normal fibroblasts was maintained at basal levels during cell proliferation but significantly increased in response to density-dependent cell contact arrest. Brautigan *et al.*(1991) reported similar results using monkey kidney epithelium CV-1 cells. Furthermore, it has been shown that the treatment of cells with orthovanadate, a PTPase inhibitor, resulted in neoplastic transformation of the NRK-1 cells which appeared not to be due to stimulated cell proliferation, but rather to the suppression of contact inhibition (Klarlund et al., 1985). Based on these observations, it is proposed that the suppression of tumorigenicity resulting from LAR overexpression in 18-Hn1-LAR cells *in vivo* could, at least in part, be due to increased cell contact inhibition, a phenomenon that was absent or greatly reduced *in vitro*.

Previous studies in our laboratory have indicated that the levels of endogenous expression of LAR and PTP1B PTPases in 18-Hn1 cells were not sufficient to confer a protein phosphorylation level as low as that observed in the non-*neu* transformed parental 184B5 cells (Zhai et al, 1993 and unpublished data). The constitutive activity of $p185^{neu}$ protein tyrosine kinase in 18-Hn1 cells (as the consequence of introduction of activated *neu* oncogene) led to hyperphosphorylation of $p185^{neu}$ and other substrates (eg. PLCγ)

in these cells and in 18-Hn1-LAR cells as well. In the current studies, immunoblot analysis shows that overexpression of LAR in 18-Hn1-LAR cells results in reduction of tyrosine phosphorylation in several cellular proteins, when compared to 18-Hn1 cells. It is conceivable that the decreased tyrosine phosphorylation observed in 18-Hn1-LAR cells (as a result of overexpression of LAR PTPase) could be the mechanism, at least in part, that explains the reduced tumorigenicity of these cells in athymic nude mice compared to control 18-Hn1 cells.

It is not clear, whether LAR acts directly on p185^{neu}-PTK, or on common substrates in the signaling pathway. A simple interpretation of these data would be that p185*neu* itself is the target substrate of LAR. It has been reported that LAR was able to dephosphorylate *in vitro* autophosphorylated EGFR (Hashimoto et al, 1992), a transmembrane PTK with extensive structural similarity to p185^{neu}. Data from our Western blot analysis, however would not support this hypothesis, as the amounts of p185^{neu} proteins were essentially identical in 18-Hn1-LAR cells and 18-Hn1 cells despite the fact that the expression of LAR was increased in 18-Hn1-LAR cells. This would suggest that the suppressed tumorigenicity of 18-Hn1-LAR cells by LAR is not due to decreased p185^{neu} expression. Alteratively, LAR-PTPase and P185neu-PTK may not simply oppose each other's action; rather, they may work in concert to maintain the balance of effector activation needed for the regulation of cell proliferation and differentiation. Thus, a second intriguing possibility exists that both p185^{neu} and LAR PTPase share common protein substrates. One of the substrates for p185^{neu} transforming potential appears to be phospholipase C-γ (PLC-γ) (Arteaga et al., 1991; Soderquist et al., 1992; Jallal et al., 1992 and Peles et al., 1991). Recent studies done by Rotin *et al.*

have demonstrated that the SH2 domain of PLC-γ was able to prevent tyrosine dephosphorylation of the EGFR and other receptor linked PTKs by RPTPγ. This suggests that the PTPase and the SH_2 domain of PLC-γ compete for the same tyrosine phosphorylation sites in the carboxyl-terminal tail of the EGFR (Rotin et al., 1992). Unpublished data from our laboratory suggests that LAR may form a complex with PLC-γ in immunoprecipitation-immunoblot analysis, implying that both $p185^{neu}$ and LAR PTPase may compete for binding to PLC-γ, presumably through the SH_2 domain. The regulation of PLCγ by $p185^{neu}$-PTK and LAR-PTPase, as well as the interaction between these proteins, could be a critical event in human breast cancinogenesis. Further examination of the pathways of $p185^{neu}$ activity and its substrate dephosphorylation by LAR will no doubt contribute to defining the roles of these enzymes in the development of human breast cancer.

A number of studies have explored the idea that the gene encoding LAR PTPase could be a potential candidate tumor suppressor gene because of its unique structure, tissue distribution and chromosome location (Fischer et al., 1991; Saito and Streuli, 1991; Streuli et al., 1988; Krueger et al., 1990, 1992; Zhai et al., 1993). In contrast to certain PTPases, which have restricted tissue distribution, LAR is expressed widely in epithelial and endothelial cells of many different organs including the mammary gland (Streuli et al., 1988, 1992; Zhai et al., 1993). In addition, the overall structure of LAR, a cell adhesion molecule (CAM) homologous extracellular domain linked to two cytoplasmic PTPase domains would suggest that the activity of this enzyme may counteract the effects of certain PTKs as well as playing an important role in contact inhibition; either activity could inhibit the tumor cell growth process (Streuli et al., 1988,

1989; Saito and Streuli, 1991). Furthermore, the extracellular region of LAR is structurally similar to the product of a putative colorectal tumor suppressor gene (DCC) (Streuli et al., 1990). DCC has been shown to suffer allelic losses in approximately 40% of human breast carcinomas (Devilee et al., 1991). In addition, the human LAR gene has been mapped to chromosome 1P32-33 (Streuli et al., 1992), a region that contains a candidate breast carcinoma suppressor gene (Genuardi et al., 1989; Weinberg, 1991). These reports, as well as results from our previous studies showing that LAR is a p185^{neu}-PTK responsive PTPase (LAR became elevated in response to *neu* oncogene transfection), provide support for the concept that LAR may act as a tumor suppressor and that its activation or regulation could be critical in human breast cancinogenesis. The data reported in this communication showing the inhibition of tumorigenicity *in vivo* by introduction of LAR cDNA into *neu* transformed human breast carcinoma cells provides compelling support for this concept. Inhibition of breast tumorigenesis by this particular PTPase or by any PTPase has, heretofore, not been reported.

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