The action of a mouse mammary tumor virus (MMTV)1 promotes oncogenes is directed primarily to the mammary gland by developed several such paradigms in which expression of various mRNAspecies whose expression pattern parallelsthemorphologicaldifferencesfoundbetweentumortypes (7).2 In general, we have found that tumors arising from ras- and neu-bearing transgenic mice fall into a common class at the molecular level and are distinguished from tumors that arise from transgenic mice bearing the c-myc transgene. The central role tyrosine phosphorylation plays in tumorigenesis has now prompted us to look specifically at genes whose products might control this process.

Tyrosine phosphorylation is a reversible process that is controlled by the opposing actions of protein-tyrosine kinases and phosphatases (PTPases) (reviewed in Refs. 8 and 9). PTPases comprise a large and complex family of related molecules that can broadly be divided into transmembranal, receptor-like molecules and non-transmembranal, intracellular ones (reviewed in Refs. 10–12). Transmembranal PTPases generally contain two PTPase catalytic domains and are thought to be capable of responding to extracellular signals. Intracellular PTPases contain one PTPase domain flanked by protein sequences that regulate the activity or localization of the entire molecule. Dual specificity phosphatases, i.e. molecules that can dephosphorylate both phosphoserine/threonine and -tyrosine exist as well. The best characterized of these, MKP1, can dephosphorylate mitogen-activated protein kinase in vivo, thereby linking this phosphatase to the latter stages of signal transduction to the nucleus (13).

Being the antithesis of tyrosine kinases, PTPases were originally thought to suppress transformation. Indeed, inhibition of PTPase activity was shown to result in transformation of cul-

Transgenic mice in which the expression of a specific oncogene is targeted to the mammary epithelium are useful models for studying the origin of breast cancer. In recent years we have developed several such paradigms in which expression of various oncogenes is directed primarily to the mammary gland by the action of a mouse mammary tumor virus (MMTV)1 promoter/enhancer. Thus, mice transgenic for MMTV-c-myc, -ras, or -int-2 develop spontaneous, generally stochastic, adenocarcinomas of the mammary gland and occasionally of other tissues as well (1–6).

Close examination of the mammary tumors obtained from MMTV-myc, -ras, and -neu transgenic mice revealed significant correlation between the distinctive histologic appearance of each tumor and the identity of its transforming oncogene (6). This correlation can be thought of as having arisen through either of two mechanisms (6, 7). One stipulates that the mammary gland contains several cell types that differ in their susceptibility to transformation by individual oncogenes; that is, each transforming oncogene targets a particular cell type that is then expanded to give rise to a distinct tumor type. An alternative hypothesis suggests that one or few mammary cell types are susceptible to oncogenic transformation, but that each oncogene drives the transformed cell along a distinct developmental pathway, imparting to it a distinctive histologic phenotype. Both mechanisms could certainly operate in whole or in part simultaneously.

The existence of oncogene-specific morphology also suggests that each type of mammary tumor expresses a somewhat different cohort of genes. The characterization of these marker genes might shed light on the details of transformation. Accordingly, we undertook to define genes expressed in particular types of mammary tumors. To date we have isolated several mRNA species whose expression pattern parallels the morphological differences found between tumor types (7).2 In general, we have found that tumors arising from ras- and neu-bearing transgenic mice fall into a common class at the molecular level and are distinguished from tumors that arise from transgenic mice bearing the c-myc transgene. The central role tyrosine phosphorylation plays in tumorigenesis has now prompted us to look specifically at genes whose products might control this process.

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1 The abbreviations used are: MMTV, mouse mammary tumor virus; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PTPase, protein-tyrosine phosphatase; RACE, rapid amplification of cDNA ends.

2 A. Elson, D. Kitsberg, and P. Leder, unpublished results.
tured rat kidney cells (14). This interpretation was strengthened by the ability of the cytoplasmic PTPases, TCPTP (15) and PTP1B (16), to suppress oncogenic transformation in cultured cells. PTP1B has also been shown to be up-regulated in a mammary cell line transformed by neu (17). Furthermore, overexpression of the dual specificity phosphatase MKP-1 has been shown to counteract some of the actions of activated ras, suggesting that it can function as an anti-oncogene (18). Nonetheless, overexpression of PTPases can in itself be transforming, as in the case of PTPβr (19), suggesting that various PTPases may be linked to transformation in different ways.

In an effort to focus on the role of tyrosine phosphorylation in mammary cell transformation, we sought PTPases that showed distinctive expression patterns in each tumor type. Here we report that the transmembrane PTPase e (20) is highly expressed in mammary tumors initiated by ras or neu, but is absent or nearly absent from mammary tumors initiated by myc or int-2. Moreover, PTPe expression appears to be mammary tumor-specific and is not found in ras-based tumors that develop in several other organs. In addition, the PTPe protein displays a tissue-specific pattern of N-glycosylation.

**EXPERIMENTAL PROCEDURES**

Transgenic Mice Lines, Tumors, and Cell Culture—Cell lines were derived from mammary gland tumors of mice carrying the MMTV-c-myc transgene (1, cell lines M158, K485, 8MA1, 11B9MA, 13MA1A, 16MB9A, MB6, and M1013), the MMTV-c-neu transgene (3, cell lines SMF, NK417, NK639, NaF, and NFF), Zeta-Globin-v-Ha-ras transgenes (AC-raline; 5, cell lines AC204, AC236, AC71, AC816), or the MMTV-int-2 transgene (4, cell line 1128). Fibrosarcoma (AC101, AC216A, and AC280) and uterine tumor (AC139) cell lines were established from tumors that arose in AC-ras transgenic mice. Samples of primary mammary or salivary gland tumors were obtained from mice transgenic for MMTV-c-myina-ras (2) or MMTV-c-myc. All cells were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

PCR Amplification—2 μg of poly(A)1 RNA purified from lines 16MB9A (MMTV-myc), SMF (MMTV-neu), or AC816 (AC-ras) was reverse-transcribed and one tenth of the reverse transcriptase product was used in each PCR amplification experiment. The following pairs of degenerate oligomers of oligonucleotides were used to amplify PTPase catalytic domains: pair AA/B, A(C/T)TT(C/T)TGG(A/G/T)(G/T)ATG(A/G)T-

AcidiGCTCTTGCTCTTCT, antisense to bases 613–593 in Fig. 5.

**RESULTS**

Identification of PTPases Expressed in Murine Mammary Tumor Cell Lines—A preliminary screen for tumor-specific PTPases was conducted in three representative epithelial cell lines previously isolated from mammary tumors initiated in transgenic mice by myc, neu, or ras. Samples of first strand cDNA from these lines were amplified by PCR using pairs of degenerate oligomer mixtures targeted at conserved sequence motifs of the PTPase catalytic domain. PCR products were then subcloned and identified by sequencing. Fragments of catalytic domains from the following seven PTPases were identified: PTPα (30), PTPγ (20), PTPγ (31), LAR (32), MPTPH3/70.15 (33), PTPH1 (34), and musCPTP (35).

PTPα mRNA is Preferentially Expressed in neu- and ras-, but Not in myc- or int-2-based Murine Mammary Tumors—The cloned PTPα fragments were used to estimate the steady-state levels of PTPα mRNA in mammary tumor cell lines. Each fragment was hybridized to blots containing RNA samples isolated from a panel of 18 epithelial cell lines previously established from mammary tumors initiated by neu, myc, ras,
or int-2. As seen in Fig. 1, PTPase e mRNA levels varied among the cell lines in a manner that correlated with the identity of the transforming oncogene. The six other PTPases listed above were uniformly expressed in all the tumor types examined and most were not studied further (results not shown).

PTPase mRNA was relatively abundant and was expressed at similar levels in all the neu- and ras-based cell lines examined. On the other hand, PTPase mRNA was either absent or present at very low levels in the MMTV-myc and -int-2 cell lines (Band A, Fig. 1). This heterogeneity of expression is characteristic of the myc-based mammary tumors and has been noted previously (7). The three additional bands present in the blot depicted in Fig. 1 (Bands B, C, and D) were subsequently found to be other forms of PTPase mRNA (results not shown).

Examination of primary tumor samples revealed that PTPase mRNA was expressed preferentially in MMTV-ras and MMTV-neu mammary tumors (Fig. 2, right panel, and not shown). This last result argues strongly that differential expression of PTPase mRNA is a characteristic of the original tumors and did not result from their adaptation to tissue culture conditions.

Up-regulation of PTPase in ras Tumors Is Mammary Gland-specific—If up-regulation of PTPase were part of a general mechanism of transformation by ras or neu, then PTPase mRNA should be up-regulated in all tumors initiated by these oncogenes. To address this point, levels of PTPase mRNA were examined in a number of ras-based tumors and cell lines derived from various tissues of mice transgenic for ras (2, 5). Significantly lower levels of PTPase RNA were found in cell lines derived from fibrosarcomas and from a uterine tumor when compared with a mammary tumor cell line (Fig. 2, left panel). Comparison of primary epithelial tumors from the salivary and mammary glands of mice transgenic for ras yielded similar results (Fig. 2, right panel). This suggests that increased levels of PTPase mRNA are not seen in all epithelial tumors. Due to lack of suitable material, similar analyses were not performed in the case of neu-initiated tumors.

Two Major PTPase mRNAs of Distinct Tissue Specificity Exist—Examination of poly(A)+ RNA from several organs revealed that the PTPase mRNA species overexpressed in mammary tumors was present in brain, heart, testes, and lung (Fig. 3, Band A). Lower levels of a slightly smaller PTPase mRNA were present in lymph nodes, thymus, lung (Fig. 3, Band B), and spleen (not shown). Virtually no expression of either PTPase mRNA was detected in liver and kidney (not shown). There was little overlap in the expression patterns of the two mRNAs, suggesting each has a specific physiological role. As will be demonstrated below, the longer mRNA species encodes the transmembranal form of PTPase (e-transmembranal). The smaller mRNA has been shown to encode a novel, non-trans-

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3 A. Elson and P. Leder, manuscript submitted for publication.
membranal mRNA while the shorter form predominated in regressing glands. In all cases, expression levels in the mammary gland and other organs were clearly well below those found in ras- and neu-based mammary tumor cell lines (Figs. 3 and 4). This indicates that the high levels of PTPε mRNA in ras and neu mammary tumors, rather than the low levels in myc tumors, were the abnormal finding.

The Longer PTPε mRNA Is the Human Homologue of the Human Transmembranal ε Phosphatase—In order to provide additional tools for understanding the role of PTPε in tumorigenesis, we cloned the ε-transmembranal cDNA from libraries prepared from the AC-816 ras-based mammary tumor cell line and from adult mouse brain. Probing was initiated with the PTPε cDNA fragment originally isolated by PCR. Sequence analysis of a number of overlapping PTPε cDNA clones revealed that the ε-transmembranal cDNA is 5412 base pairs in length, in good agreement with the position of the ε-transmembranal mRNA signal on RNA blots (Fig. 5).

The 5′-untranslated region of the ε-transmembranal cDNA is 323 base pairs long and, as in the case of the human PTPε cDNA, lacks an in-frame stop codon. The authenticity of the 5′ end was verified by performing 5′-RACE on mRNA obtained from another ras-based mammary tumor. Sequencing of several independent 5′-RACE products revealed that the 5′ ends of the ε-transmembranal cDNA were essentially as cloned from the cDNA libraries (not shown). No additional 5′ sequences were found despite extensive screening of both libraries used here. Both items, as well as cDNA expression and protein deglycosylation studies (see below), are consistent with the 5′ end of the ε-transmembranal cDNA described here being the true one.

The coding portion of the ε-transmembranal cDNA begins at an ATG codon which is part of a near perfect Kozak consensus sequence (36) and is conserved in the human PTPε (Fig. 5A and Ref. 20). The putative product of this mRNA is a 699-amino acid-long protein that has leader, extracellular, and transmembranal domains characteristic of a transmembranal protein (Fig. 5A). Hydrophobicity and sequence analyses (37) suggest that the extracellular domain of murine PTPε is 28 amino acids long. Overall, 87% of the nucleic acid sequence and 93% of amino acid residues of the murine PTPε are identical to the human form described in Ref. 20.

Examination of the relatively long 3′-untranslated sequence of the ε-transmembranal mRNA revealed the presence of seven repeats of the sequence ATTTA which is found in short-lived mRNAs (reviewed in Ref. 38). Two polyadenylation signals were also found, the distal of which was used in the set of clones examined here. This signal directed polyadenylation at either of two distinct sites, located at nucleotides 5405 and 5412 (Fig. 5, A and B).

Production and Characterization of Anti-PTPε Polyclonal Antibodies—In order to characterize PTPε expression at the protein level, polyclonal antibodies were raised against a peptide derived from the intracellular region of murine PTPε. Extracts of COS cells transiently transfected with the coding region of the ε-transmembranal cDNA served as positive controls in all the subsequent work. The anti-PTPε immune serum recognized a protein of ~105 kDa in extracts of transfected cells in immunoprecipitation and in protein blotting experiments (Fig. 6, lane 3, and results not shown). This protein was not observed in mock-transfected cells (Fig. 6, lane 1), nor when the experiments were repeated with preimmune serum or with immune serum in the presence of an excess of the immunizing peptide (Fig. 6, lane 2; Fig. 7, right panel, and not shown). Taken together, these results show that the antisera was capable of recognizing the ε-transmembranal protein. The observed size of PTPε in these experiments was larger than its predicted molecular mass of ~80 kDa. This fact as well as its migration as a broad band indicated that the ε-transmembranal protein is glycosylated, as is demonstrated below.

Mammary Tumor Type-specific Expression of the PTPε Protein—Protein blots prepared from homogenates of several representative mammary tumor cell lines were probed with anti-PTPε serum (Fig. 7). The ε-transmembranal protein was detected as a broad band of ~105 kDa in extracts from MMTV-ras and MMTV-neu cell lines. In agreement with RNA data presented in Fig. 1, the ε-transmembranal protein was absent from the representative MMTV-myc cell line used here (Fig. 7, left panel). This result indicates that the expression of the transmembranal form of PTPε is indeed limited to ras- and neu-based murine mammary tumors. Interestingly, a protein band corresponding to the shorter, non-transmembranal isoform of PTPε was present at relatively low levels in extracts from the MMTV-myc cell line (Fig. 7, left panel). The particular myc cell line used here does indeed express low levels of the mRNAs of both PTPε isoforms (Fig. 1, lane 10). Note that despite its presence, the ε-transmembranal mRNA present at low levels in this myc cell line does not give rise to mature ε-transmembranal protein.

Expression of Electrophoretically Distinct Forms of PTPε in Brain and Lungs—We next examined the forms of the PTPε protein present in normal mouse tissues. In agreement with RNA data (Fig. 3), the ε-transmembranal protein was detected as a broad band in protein extracts of brain and lung (Fig. 7, left panel). Interestingly, ε-transmembranal protein from lung and brain were of distinct sizes. An additional broad band corresponding to a protein of ~140–160 kDa was specifically recognized by the anti-PTPε serum in both of these tissues (Fig. 7, left panel, upper-most band). This band was not as prominent in the mammary tumor cell lines. Cellular fractionation experiments reveal that this protein is membrane-associated (not shown), and its migration as a broad band suggests it is glycosylated. This protein may represent yet another form of the ε-transmembranal protein or a cross-reactive form of another protein.

The PTPε Protein Is N-Glycosylated in a Tissue-specific Manner—Detection of the ε-transmembranal protein as a broad band of larger than expected size in protein blots suggested that it is glycosylated. In order to address this point protein extracts of a ras-based mammary tumor cell line were treated with a variety of glycosidases (Fig. 8). Treatment with neuraminidase or with endo-α-N-acetylgalactosaminidase did not appear to significantly affect the mobility of the ε-transmem-
branal protein (Fig. 8, lanes 2 and 5). On the other hand, removal of N-linked carbohydrates by N-glycosidase-F resulted in a significant reduction in the apparent size of the molecule (Fig. 8, lanes 3 and 4). Most of the protein was now found in two sharp, closely spaced bands; 80 kDa, in good agreement with the predicted mass of the e-transmembrane protein. The same results were obtained when the extracts were treated with a mixture of all three deglycosylating enzymes (Fig. 8, lane 6). The heavier 140-kDa band present in the extracts was virtually unaffected by neuraminidase and endo-α-N-acetylgalactosaminidase; it exhibited only a slight decrease in size after treatment with N-glycosidase-F. We believe that it is glycosylated in a way that is resistant to the actions of the deglycosylating enzymes used here.

Similar experiments were repeated with protein extracts from lung and brain (Fig. 8, right panel). As in the case of the ras mammary tumor, removal of N-linked carbohydrates by N-glycosidase-F had the most significant effect on the mobility of the expressed proteins in these tissues.

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**Fig. 5.** The murine PTPε cDNA. A, cDNA and translated protein sequence of the transmembrane form of PTPε (Band A in Figs. 1–4). The signal peptide and the transmembrane domain are underlined. Two N-linked glycosylation sites, the peptide used for generation of antisera, and the first two ATTTA motifs are boxed. Most of the 3′-untranslated region is not shown. B, schematic representation of the entire cDNA of PTPε. Thick and thin rectangles denote coding and untranslated regions, respectively. Thick black box denotes transmembrane domain; thick shaded boxes denote the PTPase catalytic domains. Thin black boxes mark ATTTA signals and thin boxed shaded boxes denote the polyadenylation signals.
of the \(\varepsilon\)-transmembranal protein. Note that the significant differences in the apparent molecular mass of the glycosylated \(\varepsilon\)-transmembranal proteins of lung and brain disappeared after deglycosylation (compare Fig. 7, left panel, with Fig. 8, lanes 10 and 12). This indicates that the differences in the electro-phoretic mobility of the \(\varepsilon\)-transmembranal proteins of lung and brain reflect tissue-specific glycosylation patterns. Note also that the apparent masses of the deglycosylated forms of the \(\varepsilon\)-transmembranal protein from lung and brain were slightly different from that of the mammary tumor (Fig. 8, lanes 10–12). This raises the possibility that other post-translational modifications, such as phosphorylation, are still present on the deglycosylated \(\varepsilon\)-transmembranal protein.

**DISCUSSION**

Our results indicate that there is a dramatic increase in expression of PTP\(\varepsilon\) in ras- and neu-based mammary tumors, both at the mRNA and protein levels. Mammary tumors initiated by these two oncogenes in fact form a class that specifically expresses a cohort of marker genes which are underexpressed or silent in mammary tumors initiated by myc and int-2 (7). This finding can be accounted for by assuming that the ras and neu oncogenes, acting in the context of the mammary epithelial cell, share a common transformation mechanism and/or transform a common susceptible target cell. Interestingly, neu and ras have been suggested to act through a common pathway (7, 39–41) which may serve as the basis for up-regulating PTP\(\varepsilon\) and other marker genes.

The possibility that PTP\(\varepsilon\) is an invariant link in the ras transformation pathway appears less likely in view of the fact that relatively low levels of PTP\(\varepsilon\) mRNA are found in several ras-based tumors that arise in non-mammary tissues, including some of epithelial origin. Thus it seems that overexpression of PTP\(\varepsilon\) in ras-based tumors is at least partially specific to mammary tumors and is not part of a general mechanism by which ras and neu transform. Nonetheless, it remains possible that PTP\(\varepsilon\) is part of a more restricted mammary-specific transformation pathway.

Bearing this point in mind, it is important to note that the isoform of PTP\(\varepsilon\) up-regulated in these mammary tumor cells is transmembranal and therefore can be conceptually integrated into the mechanisms by which neu and other tyrosine kinases act. A similar integration is possible in the case of ras, whose association with the cellular membrane is critical for its transforming ability (42, 43). Moreover, ras influences the expression of genes whose products are thought to act upstream of itself in signal transduction pathways. For example, ras-mediated transformation has recently been shown to affect expression of the transcription factor AP2 (44) which in turn regulates neu expression (45).

Examination of the extracellular domain of transmembranal PTP\(\varepsilon\) protein reveals two sites for N-linked glycosylation and a number of serine and threonine residues that could be substrates for O-linked glycosylation. Indeed, the transmembranal isoform of PTP\(\varepsilon\) is present in various cell types as an N-linked glycoprotein of 100–110 kDa that is analogous to a 100-kDa form of the closely-related \(\alpha\)-phosphatase which is also predominantly N-glycosylated (29).

In a way that creates an opportunity for differential function, each tissue or tumor examined displays a unique pattern of PTP\(\varepsilon\) glycosylation. Tissue-specific glycosylation arises from differences in the glycosylating capabilities of tissues rather than from a particular property of the glycosylated protein (46). Nonetheless, the precise glycosylation pattern of PTP\(\varepsilon\) could affect its function by, for example, influencing proteins that interact with its extracellular domain. The electrophoretic mobilities of PTP\(\varepsilon\) from the mammary tumors and from normal mammary tissue are identical (not shown), indicating that their glycosylation patterns are similar. The precise pattern of glycosylation of PTP\(\varepsilon\) in the mammary tumors may therefore be mammary gland- rather than tumor-specific.

Finally, it is worth noting that the structurally related PTP\(\alpha\), PTP\(\varepsilon\) is the only PTPase known to have transforming ability when overexpressed (19). PTP\(\varepsilon\) and PTP\(\alpha\) are both type IV PTPases and are the only known members of this class (10, 12). Given that PTP\(\alpha\) displays the highest degree of sequence and structural similarity to PTP\(\varepsilon\), it is possible that PTP\(\varepsilon\) may share some of the transforming abilities of PTP\(\alpha\) and that
up-regulation of PTPε in mammary tumors is indeed linked to the transformation process. Subsequent efforts, involving the creation of transgenic mice, will be directed toward testing this possibility.

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