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Protein-Tyrosine Phosphatases Expressed in Mouse Epidermal Keratinocytes

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The importance of growth factors acting via receptor-type protein-tyrosine kinases in the continuous renewal of the epidermis from the keratinocyte stem cell population has been well established. Protein-tyrosine phosphatases (PTPases), which dephosphorylate phosphotyrosine-containing proteins, may therefore be expected to play an equally important role in the control of epidermal growth and differentiation. In this study, we have made an inventory of the various PTPases that are expressed during mouse keratinocyte proliferation and maturation. A panel of 13 different PTPase probes was obtained by combining a set of PTPase cDNAs previously cloned from mouse brain and a set of PTPase probes obtained from a normalized keratinocyte PTPase cDNA library. This PTPase cDNA panel, spanning probes for receptor-type as well as cytoplasmic-type family members, was used to monitor RNA expression levels in keratinocyte fractions isolated from murine epidermis and in keratinocyte cell cultures. No overt changes were observed in PTPase mRNA levels in all strata of mouse epidermis, but comparison of cultured cells with freshly isolated keratinocytes revealed several conspicuous differences. In the cultured Balb/MK cell line, absence of PTPδ expression and upregulation of PTPcg and, to a lesser extent, PTPγ mRNA ratios were observed compared to the freshly isolated cells. These results provide a basis for further research on the impact of PTPase activity on epidermal growth control. Key words: differentiation/mouse/phosphotyrosines/signal transduction. J Invest Dermatol 106:972-976, 1996

Epidermis is continuously renewed from a germinative cell population in the basal layer (Fuchs, 1990). In normal skin most of the keratinocytes in this layer are quiescent, and only about 10% are involved in cell division (Jones and Watt, 1993). Under hyperproliferative conditions, as in wound repair or in psoriatic lesions, the quiescent keratinocytes are recruited into the cell cycle. Keratinocytes produced in the basal layer subsequently enter a differentiation program that leads to keratinization and, finally, cell death (Fuchs, 1990). Accumulating evidence indicates that phosphorylation of tyrosine residues is a critical step in this series of events. Protein phosphotyrosine turnover rates in epidermal cells, for example, reflect their mitotic index (Gentleman et al, 1984). The involvement of protein tyrosine kinases (PTKs) in keratinocyte proliferation has also become evident from studies using tyrphostin tyrosine kinase inhibitors (Dvir et al, 1991). In particular, growth factors that bind to cell surface receptors containing cytoplasmic tyrosine kinase domains (Hunter et al, 1992), including epidermal growth factor (EGF), keratinocyte growth factor, transforming growth factor α, β fibroblast growth factor, and insulin-like growth factor I, have been shown to be potent stimulators of keratinocyte growth (Ristow and Messner, 1988; Elder et al, 1989; Aaronson et al, 1990; Krane et al, 1991, 1992). In hyperproliferative areas of the skin, the activity of the EGF receptor is indeed markedly increased (Krane et al, 1992; Yates et al, 1991). Tyrosine phosphorylation also appears to be an early and specific event in keratinocyte differentiation (Filvaroff et al, 1990). In cultured keratinocytes, the cytoplasmic PTK Src is normally myristoylated and anchored in the perinuclear and plasma membranes. Upon treatment with calcium and ionophores to induce differentiation, Src is activated and is released into the (peri)nuclear region of differentiating keratinocytes (Zhao et al, 1992). Indeed, the import of Src into the nucleus parallels the overall increase in nuclear phosphotyrosine content of differentiating keratinocytes.

Phosphotyrosine levels in the cell are determined by the balanced action of protein tyrosine kinases and protein tyrosine phosphatases (PTPases) (Hunter et al, 1992; Walton and Dixon, 1993). Therefore, PTPases must also play an important role in controlling epidermal growth. Indeed, phosphotyrosine phosphatase activity was found to be significantly greater in psoriatic lesions compared to normal skin (Gentleman et al, 1984). More recently, Gunaratne et al have demonstrated that the prototype cytoplasmic tyrosine phosphatase PTP1B is capable of dephosphorylating several substrates of the EGF receptor, including the receptor itself, in vitro (Gunaratne et al, 1994). Moreover, PTP1B protein and the EGF receptor were shown to be similarly restricted to the basal/spinous compartment in epidermal skin, suggesting a role for PTP1B in the in vivo regulation of epidermal functions (Gunaratne et al, 1994).

Here we describe the characterization of PTPase family members...
expressed in keratinocytes employing degenerate oligodeoxynucleo-
otide primers and polymerase chain reaction (PCR) techniques in
order to obtain a better appreciation on the role of reversible
tyrosine phosphorylation in epidermal growth and differentiation.
Moreover, the same approach was used to monitor PTase expres-
sion at the RNA level in keratinocytes isolated from mouse
epidermis and in cultured cells.

MATERIALS AND METHODS

Cell Lines and Tissues. Culture conditions for the nontransformed
keratinocyte line Balb/MK (Weissman and Aronson, 1983) were as
described (van Houwelingen et al, 1993). Exponentially growing cells at
30–40% confluence were rendered quiescent by replacing complete in-
duum with maintenance medium (van Houwelingen et al, 1993). Differentiated
Balb/MK cells were generated by the addition of Ca++ (1.2 mM final
concentration) to complete medium and a further 2 days of incubation.
Mouse 3T3 cells were cultured according to Rinehart and Green (1975).

Fractionation of mouse epidermis was according to Furstemberger et al
(1986). Skin was taken from the back of neonatal mice (strain NMRI,
Institut für Versuchstierkunde, Hannover, Germany) and floated overnight
on 0.25% trypsin in phosphate buffered saline without calcium and magne-
sium, at 4°C. Epidermis was separated from dermis, and keratinocytes were
removed from the horny layer by gentle movement. The washed cell
suspension was layered on top of a discontinuous Percoll gradient and
centrifuged at 22°C for 20 min at 3,200 rpm. Visible fractions I and II,
resembling the less dense, nonviable, and differentiating cells from the
stratum basale, were collected.

stratum granulosum and stratum spinosum, and fractions III, Illa, and IV,
containing viable cells from the stratum basale, were collected.

Construction of Normalized PTase Library. Single-stranded
cDNA, generated by reverse transcription of 2 µg of mRNA isolated from
exponentially growing Balb/MK cells, was used as template in a PCR
reaction employing primers 1 and 2 as described above. Excess primers were
removed by Centricron-30 filtration. The cDNA was suspended in
0.12 M sodium phosphate (pH 6.8), 0.1% SDS. Double-stranded DNA was
cloned in 0.25 M sodium phosphate (pH 6.8), 0.1% SDS, Column
eluates were desalted and concentrated to 75 µl in TE buffer (10 mM
Tris-HCl, pH 8.0/1 mM ethyleneimine tetrasacetic acid (EDTA)) by
Centricon-30 and quick chilling on ice, 1

for 45 s, and at 72°C for 1.5 min.

random hexamers (Pharmacia, Piscataway, NJ). After hearing for 10 min at
room temperature. Following neutralization by
addition of an equal volume of 2 M NaHAc, 100 µl (10 µg of denatured
plasmid) was loaded per well. After washing the wells twice with 100 µl of 1
M NaHAc, the DNA was UV-crosslinked to the membrane (Stratallinkera,
Strategene, La Jolla, CA). PTase cDNA fragments, generated by RT-PCR,
employing primers 1 and 2 (see above), were loaded on a 1.5% low-gelling
temperature agarose gel. Appropriately sized products (around 350 bp)
were excised and labeled radioactively by random priming (Feinberg and
Vogelstein, 1983). Hybridization conditions were those of Church and
Gilbert (1984). After a prehybridization in 7% SDS, 0.5 M sodium
acetate, at 4°C, the membranes were hybridized with the probe pool and
were not investigated further.

RESULTS AND DISCUSSION

To investigate the diversity of protein–tyrosine phosphatase expres-
sion in keratinocytes, mRNA was isolated from Balb/MK cells in
exponential phase and used as a template for cDNA synthesis from
random hexamer primers. The resulting cDNAs served as templates in
PCR reactions using degenerate oligonucleotide primers previously
used to isolate cDNA fragments from ten different PTases expressed
in mouse brain (Hendriks et al, 1995a). The population of
double-stranded PTase cDNA fragments was then subjected to a
normalization procedure based on denaturation and incomplete
reannealing, followed by the separation of single- and double-
stranded DNA populations (Patanjali et al, 1991) to enrich for rare
transcripts. The single-stranded fraction, containing relatively low
levels of common PTase cDNAs, was then amplified using a
second set of degenerate primers (primers 2 and 3) and cloned.

More than 100 clones were screened with a mixture of 10
previously identified mouse PTase cDNAs, comprising the recep-
tor-type PTases PTPβ, LRP, LAR, PTP5, BPTP1 and PTPε, the
cytosolic PTP1B, and three novel PTPases, mPTP38 (Hendriks
et al., 1994). Briefly, two sheets of Whatmann 3MM paper overlaid by a
nylon membrane (Hybond-N*; Amersham, Arlington Heights, IL) were
moistened in water and placed in a Milliblot-D dot blot apparatus (Waters
Associates, Milford, MA). Wells were washed once with 100 µl of 1
M NaHAc. The plasmid DNA (100 ng) was incubated in 500 µl of 0.2
N HAc for 10 min at room temperature. Following neutralization by
addition of an equal volume of 2 M NaHAc, 100 µl (10 µg of denatured
plasmid) was loaded per well. After washing the wells twice with 100 µl of 1
M NaHAc, the DNA was UV-crosslinked to the membrane (Stratallinkera,
Strategene, La Jolla, CA). PTase cDNA fragments, generated by RT-PCR,
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Gilbert (1984). After a prehybridization in 7% SDS, 0.5 M sodium
acetate, at 4°C, the membranes were hybridized with the probe pool and
were not investigated further.

Two negative clones were picked at random and sequenced (Fig
1). One clone was found to contain a sequence encoding part of the
first phosphatase domain of mouse PTPy (Brame et al, 1993). Four
others contained sequences identical to the published sequence of
PTpε (Jiang et al., 1993), while seven clones were derived from
p19–PTP transcripts (den Hertog et al, 1992). The majority of the
other negative clones hybridized with the three new PTase
fragments, while the remaining negative clones proved to contain
PCR artifacts. The power of the normalization technique for the
selection of rare transcripts from the mouse keratinocyte PTPase
library is clearly demonstrated by our isolation of three additional
cDNA fragments mPTPγ-1, mPTPγ-2, and in particular mPTP–
P, whose corresponding mRNA is almost undetectable in Balb/MK cells (see Fig 2).

To monitor the relative expression levels of these different
PTase members in epidermal cell layers we used a PCR-based
protocol (Hendriks et al, 1994). Briefly, RNA isolates were used as
template in a reverse transcription (RT) reaction, and the different

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A water-jacketed Bio-Gel HTP
Demin was maintained at 60°C as were the buffers used for
analysis. Single-stranded DNA was eluted from the column
using 0.12 M sodium phosphate (pH 6.8), 0.1% SDS. Double-stranded
cDNA was eluted in 0.25 M sodium phosphate (pH 6.8), 0.1% SDS, Column
Nucleotide sequences and deduced amino acid sequences of three PTPase cDNA fragments obtained from a normalized mouse Balb/MK and 3T3 cells was that both LAR and PTP14 were detected in the keratinocytes but not in the fibroblasts. This is in agreement with RNA in situ hybridization studies on PTP14 (Hendriks et al., 1995b) and immunohistochemical studies on LAR (Streuli et al., 1992) that show both phosphatases to be expressed in epithelial tissues. No expression of PTP8 was detected in the cell lines, but PTP8 expression was readily detected in all of the mouse epidermal layers, and was found to be the most abundant PTPase in mouse brain. The limited data available from the literature describe PTP8 expression in neuronal cells and in the B-cell lineage (Mizuno et al., 1993). The observed expression of PTP8 in the epidermis may be attributable to the presence of melanocytes or Langerhans cells. Determination of the PTPase expression pattern in human primary melanocytes using the same RT-PCR approach, however, revealed a very weak PTP8 signal, just above background (Hendriks and van Muijen, unpublished). In addition, Langerhans cells are isolated by Ficoll separation at densities below that of fraction IV (Sullivan et al., 1985), a keratinocyte fraction that displays a clear PTP8 signal. Further, keratinocyte cultures (Sutter et al., 1991) exhibit considerable PTP8 expression (Fig 2). The expression of PTP8 in the keratinocyte cells.

Another striking difference between PTPase expression patterns in the keratinocyte cell line and the epidermal cell layers was the much higher expression of PTPk in Balb/MK cells. This could result from the conditions of tissue culture, because 3T3 cells also exhibit considerable PTPk expression (Fig 2). The expression of PTPk has been reported to be widespread, with especially high PTPk levels being found in liver and kidney tissue (Jiang et al., 1993). It is not currently known, however, in which particular cell types PTPk is expressed, nor is it clear whether its expression is developmentally regulated. Interestingly, the observed differences in expression of PTP8 and PTPk in freshly isolated cells and keratinocyte cell lines parallels the suppression of keratins K1 and K10, and the concomitant induction of keratins K6 and K17 in keratinocyte cultures (Sutter et al., 1991). It is tempting to speculate that the expression of PTP8 and PTPk may be subject to the same regulatory controls as these keratins. The Balb/MK cell line was established from keratinocytes of 24- to 48-h-old mice (Weissman et al., 1985).
Figure 2. Dot-blot analysis of labeled PTPase PCR fragments amplified from various sources, on filters carrying equimolar amounts of specific cDNA clones. The leftmost panel on the top shows the order in which the clones were spotted. Delta, mPTPβ1; BPTP, mBPTPl; LAR, mLAR-1; Epillon, mPTPβ-1; LRP, mLRP-1; Beta, pBluescript; GAPDH, rat glyceroldehyde phosphate dehydrogenase cDNA; Gamma, mPTPβ-1; Kappa, mPTPβ-1. PTPase cDNA fragments, except mP19PTP, mPTPβ-1, and mPTPβ-1, have been described elsewhere (Hendriks et al, 1995a). The results with PTPase probes derived by RT-PCR on RNA isolated from quiescent (Qui), exponentially growing (Exp), and in vitro differentiated (Diff) mouse balb/MK cells, as well as from 3T3 fibroblasts (3T3) are shown at the top of the figure. Results obtained using RNA isolates from different fractions (I, II, III, and IV) of mouse skin cells (Furstenberger et al, 1986) are shown in the lower part of the figure. The pattern obtained with mouse brain RNA is shown on the right (Brain) for comparison. pBluescript was used as a probe to confirm equal loading (data not shown). A GAPDH clone and the empty vector were spotted to reveal background hybridization levels.

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and Aaronson, 1983). Perhaps a lack of signals from other epidermal cell types or from the dermis made the Balb/MK cells lose their PTPβ expression. Our current experiments are aimed at investigating these possibilities. The identification of specific PTPases expressed in the keratinocyte cell line and in freshly isolated cells provides an insight into the identity of those enzymes that potentially regulate intracellular phosphotyrosine levels and thereby influence epidermal growth and differentiation processes. For PTPβ1, it has been demonstrated that it can dephosphorylate epidermal phosphotyrosine-containing proteins in vitro (Guanzato et al, 1994). Also the fact that its distribution in skin parallels that of the EGFR suggests a role in epidermal growth control (Guanzato et al, 1994). Another PTPase expressed in keratinocytes, LRP (Matthews et al, 1990), also known as PTPα (Krueger et al, 1990), has been shown to activate Src in several cell types (Zheng et al, 1992; den Hertog et al, 1993). LRP may exert a similar effect on Src in keratinocytes which could result in the redistribution of Src to the nucleus and eventually in keratinocyte differentiation (Zhao et al, 1992). Clearly, experiments to modulate the levels and activities of the various PTPase protein family members will be required to reveal their impact on normal keratinocyte growth and differentiation and their potential role in epidermal disorders such as psoriasis and skin cancers.


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