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 PTPκ 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/phosphotyrosine/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 (Ristroph 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 oligodeoxynucleotide 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 PTPase expression 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 Aaronson, 1983) were as described (van Hoofdik et al., 1993). Exponentially growing cells at 30–40% confluence were rendered quiescent by replacing complete medium with maintenance medium (van Hoofdik et al., 1993). Differentiated Balb/MK cells were generated by the addition of Ca²⁺ (1.2 mM final concentration) to complete medium and a further 2 days of incubation. Mouse 3T3 cells were cultured according to Rheinwald and Green (1975).

Fractionation of mouse epidermis was according to Fürstenberger 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 magnesium, 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, representing the less dense, nonviable, and differentiating cells from the stratum granulosum and stratum spinosum, and fractions III, Illa, and IV, containing viable cells from the stratum basale, were collected.

cDNA Synthesis and PCR Amplification: Total RNA was isolated using the LiCl/Urea method (Ausubel and Roazen, 1980). PTPase cDNA fragments were generated by reverse transcriptase (RT)-PCR as described earlier (Hendriks et al., 1995a) with minor modifications. Briefly, RNA (2 μg) was pelleted and dissolved in 17.5 μl of sterile water containing 2 μg of random hexamer primers (Pharmacia, 1 μl of RNasin (40 U/μl; Promega), 3 μl of 0.1 M diithiothreitol, 1.5 μl of dNTP-mix (10 mM of each dNTP; Pharmacia), 6 μl of 5×Superscript RT buffer, and 1 μl of Superscript (RNaase H) reverse transcriptase (200 U/μl; BRL, Bethesda, MD) was added. The RT reaction mixture was kept at 42°C for 1 h. Following a 5-min incubation at 90°C, 5 μl of the single-stranded cDNA preparation was then used as template for PCR.

Degenerate oligodeoxynucleotide primers 1 and 2, based on consensus sequences from highly conserved amino acid stretches within the catalytic domains in PTPases, were as described previously (Hendriks et al., 1995). This primer pair yielded products of about 350 bp. For use in the normalization procedure, degenerate primer 3 (5'-AA(A/G)TG(C/T)G(A/C)(A/G/C/T)C(A/A)G(C/G/C/T)C(T/G)(A/G)(C/G/C/T)/ C(A)/(C/G/C/T)(A/G)/(C/G/C/T)TGCCC-3') was chosen within this 350-bp segment to yield, in combination with primer 2, fragments of about 300 bp.

Primers (final concentration of 7 ng/μl) were added to a 100-μl reaction mixture containing 50 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 0.01% bovine serum albumin, all four dNTPs (each at 250 μM), 2 units of Taq Polymerase (Perkin-Elmer Cetus, Emeryville, CA), and template material. Thirty-five cycles were performed on a Perkin-Elmer Thermal Cycler; each cycle involved an incubation at 94°C for 45 s, at 37°C for 45 s, and at 72°C for 1.5 min.

Construction of Normalized PTPase 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 Centricon-30 filtration (Amicon, Beverly, MA). Normalization, according to Patanjalii et al. (1991), was as follows. A 50-μl reaction mixture containing 0.3 M sodium phosphate (pH 6.8), 0.4 mM EDTA, 0.04% sodium dodecyl sulfate (SDS), and 1 μg of the PTPase fragment DNA was overlaid with 0.5 ml of mineral oil and heat-denatured in a boiling water bath for 5 min. Reassociation of the DNA proceeded for 8 h at 65°C before the addition of 1 ml of 0.01 M sodium phosphate (pH 6.8), 0.1% SDS, and chilling on ice. The sample was applied to a water-jacketed Bio-Gel HTP hydroxypatite column (Bio-Rad, Richmond, CA) with a bed volume of 0.5 ml in 0.01 M sodium phosphate (pH 6.8). 0.1% SDS and a flow rate of 6 ml/h. The column was maintained at 60°C as were the buffers used for binding and elution. Single-stranded DNA was eluted from the column using 0.12 M sodium phosphate (pH 6.8), 0.1% SDS. Double-stranded DNA was eluted 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 ethyleneamine tetraacetic acid (EDTA)) by Centricon-30 filtration. A 10-μl aliquot of the single-stranded fraction was used in a 100-μl PCR reaction employing primers 2 and 3 as described above.

RESULTS AND DISCUSSION

To investigate the diversity of protein-tyrosine phosphatase expression 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 PTPases expressed in mouse brain (Hendriks et al., 1995a). The population of double-stranded PTPase 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 (Patanjalii et al., 1991) to enrich for rare transcripts. The single-stranded fraction, containing relatively low levels of common PTPase 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 PTPase cDNAs, comprising the receptor-type PTPases PTPβ, LRP, LAR, PTPδ, BPTP1 and PTPε, the cytoplasmic PTP1B, and three novel PTPases, mPTP13, mPTP14 and mPTP38 (Hendriks et al., 1995a). One third of the clones hybridized with the probe pool and were not investigated further. Twelve 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 (Barna et al., 1993). Four other contained sequences identical to the published sequence of PTPα (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 PTPase 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, mPTPε-1, and in particular mPTPα-1, whose corresponding mRNA is almost undetectable in Balb/MK cells (see Fig 2).

To monitor the relative expression levels of these different PTPase 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
PTPase cDNAs were amplified with degenerate primers and PCR. The resulting products were labeled and used as a probe on dot-blot containing equimolar amounts of the cloned mouse PTPase cDNA fragments. Using nonsaturating amounts of probe DNA, the resulting signal intensities reflect the relative abundance of the individual mRNAs in the starting material.

Five different keratinocyte populations were isolated from the back skin epidermis of neonatal mice by density gradient centrifugation (Fürstenberger et al., 1986) and used for total RNA isolation and subsequent RT-PCR. The same PTPase RT-PCR protocol was performed using RNA isolated from quiescent, exponential phase, and differentiated Balb/MK cells, to allow comparison of the PTPase expression data from freshly isolated keratinocytes with keratinocyte cell lines. In addition, RNA from 3T3 fibroblasts was taken to assess PTPase expression in dermal cells, while mouse brain RNA was also included to establish the tissue specificity of the 13 different PTPases assayed, including changes in protein levels, subcellular localizations, and differential expression patterns across different cell lines. 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., 1992). 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 Ficol separation at densities below that of fraction IV (Sullivan et al., 1985), a keratinocyte fraction that displays a clear PTP8 signal. Therefore, we conclude that the detection of PTP8 mRNA in epidermal cell fractions of widely different specific densities reflects PTP8 expression in the keratinocyte.

Another striking difference between PTPase expression patterns in the keratinocyte cell line and the epithelial cell layers was the much higher expression of PTPx in Balb/MK cells. This could result from the conditions of tissue culture, because 3T3 cells also exhibit considerable PTPx expression (Fig 2). The expression of PTPx has been reported to be widespread, with especially high PTPx levels being found in liver and kidney tissue (Jiang et al., 1993). It is not currently known, however, in which particular cell types PTPx is expressed, nor is it clear whether its expression is developmentally regulated. Interestingly, the observed differences in expression of PTP8 and PTPx in freshly isolated cells and keratinocyte cell lines parallels the suppression of keratin 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 PTPx 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., 1995).
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and Aaronsen, 1983). Perhaps a lack of signals from other epidermal cell types or from the dermis made the Balb/MK cells lose their PTP 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 PTP1B, it has been demonstrated that it can dephosphorylate epidermal phosphotyrosine-containing proteins in vitro (Gunaratne et al, 1994). Also the fact that its distribution in skin parallels that of the EGF receptor suggests a role in epidermal growth control (Gunaratne et al, 1994). Another PTPase expressed in keratinocytes, LRP (Matthews et al, 1990), also known as PTPx (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 nucleoplasm 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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