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 PTPγ expression and upregulation of PTPα 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 tyrhostin 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 ionshores 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** Cell culture conditions for the nontransformed keratinocyte line Balb/MK (Weissman and Aaronson, 1983) were as described (van Hoofdijk et al., 1993). Exponentially growing cells at 30–40% confluence were rendered quiescent by replacing complete medium with maintenance medium (van Hoofdijk 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 Rinearnd 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, Ila, and IV, containing viable cells from the stratum basale, were collected.

dDNA Synthesis and PCR Amplification Total RNA was isolated using the LiCl/Urea method (Auffray and Ragozen, 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 hexamers (Pharmacia, Piscataway, NJ). After heating for 10 min at 65°C, the sample was applied to a 100-μl PCR reaction employing primers 2 and 3 as described above. 17.5 μl of sterile water containing 2 μg of mRNA isolated from Balb/MK cells was used as template for PCR. 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 Rinearnd and Green (1975).

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 Centricron-30 filtration, and the single-stranded DNA was then amplified using a PCR-based normalization procedure based on denaturation and incomplete annealing, 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 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 PTPase families (Hendriks et al., 1995a). The population of double-stranded PTPase cDNA fragments was then subjected to a normalization procedure based on denaturation and incomplete annealing, 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 PTPase cDNAs, was then amplified using a second set of degenerate primers (primers 2 and 3) and cloned.

RESULTS AND DISCUSSION

To investigate the diversity of protein-tyrosine phosphate 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 annealing, 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 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 PTPγ (Barme et al., 1993). Four others contained sequences identical to the published sequence of PTPγ (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 mPTPy-1, mPTPK-1, and in particular mPl 9, 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-bLOTS 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 aubergine, excretion, 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 PTPase expression. The results, presented in Fig 2, show that, for the 13 different PTPases assayed, no major changes in mRNA levels occur during differentiation either in cultured keratinocytes or in freshly isolated cells. We cannot exclude, however, the possibility that other PTPases do show altered expression patterns during differentiation, because the design of the degenerate primers limits the survey to a restricted subset of the PTPase family. Moreover, factors potentially of importance to the differentiation program, such as changes in protein levels, subcellular localizations (Mauro and Dixon, 1994), or specific activities (Hunter et al., 1992) were not monitored in our assay.

An important difference in the pattern of PTPase expression in Balb/MK and 3T3 cells was that both LAR and PTP14 were not monitored in our assay. The PCR primer sequences (corresponding to the conserved stretches ‘KCDQYWP’ and ‘HCSAGVGR.’; see Hendriks et al., 1995a) are not included and the numbering is based on the published full-length mouse cDNAs (den Hertog et al., 1992; Barnen et al., 1993; Jiang et al., 1993).

Figure 1. Nucleotide sequences and deduced amino acid sequences of three PTPase cDNA fragments obtained from a normalized mouse keratinocyte PTPase cDNA library. The PCR primer sequences (corresponding to the conserved stretches ‘KCDQYWP’ and ‘HCSAGVGR.’; see Hendriks et al., 1995a) are not included and the numbering is based on the published full-length mouse cDNAs (den Hertog et al., 1992; Barnen et al., 1993; Jiang et al., 1993).
Figure 2. Dot blot analysis of labeled PTase 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, mPTP-1; BPTP, mBPTP; LAR, mLAR-1; Epilson, mPTPase-1; LRP, mLRP-1; strret, pBlue- script SK + ; P19-PTP, mP19PTP; PTP1B, mPTP1B; IIII, mPTP13; IV, mPTP14; J, mPTP38; Beta, mPTPase; GAPDH, rat glyceraldehyde phosphate dehydrogenase cDNA; Gamma, mPTPase-1; Kappa, mPTPase-1. PTase cDNA fragments, except mP19PTP, mPTP1, and mPTP1-4, have been described elsewhere (Hendriks et al., 1995a). The results with PTase probes derived by RT-PCR on RNA isolated from quiescent (Qui), exponentially growing (Exp), and in vitro differentiated (Dif) 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, IIIa, and IV) of mouse skin cells (Fürstenberger 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.

and Aaronson, 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 PTases 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 (Guranatnae et al., 1994). Also the fact that its distribution in skin parallels that of the EGF receptor suggests a role in epidermal growth control (Guranatnae et al., 1994). Another PTase 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 PTase 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.

Rastow HJ, Monner TO: Basic fibroblast growth factor and insulin-like growth factor 1 are strong mitogens for cultured mouse keratinocytes. J Cell Physiol 137:277–284, 1988


