Protein-Tyrosine Phosphatases Expressed in Mouse Epidermal Keratinocytes

Wiljan Hendriks, Curt Brugman, K. Hartmut Richter,‡ Candida van Hooijdonk,‡ Jan Schepens, Joost Schalkwijk,‡ and Bé Wieringa

Department of Cell Biology & Histology and ‡Department of Dermatology, University of Nijmegen, Nijmegen, The Netherlands; and †Department of Tissue Specific Regulation, German Cancer Research Center, Heidelberg, Germany

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 PTPα 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/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, Ssrc is activated and is released into the (per)nuclear region of differentiating keratinocytes (Zhao et al, 1992). Indeed, the import of Ssrc 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 differentiation and epidermal growth. Moreover, the same approach was utilized to monitor PTase 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 Hoofdonk et al, 1993). Exponentially growing cells at 30–40% confluence were treated quiescent by replacing complete medium with maintenance medium (van Hoofdonk 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 Ritesh et al (1986). Skin was taken from the back of neonatal mice (strain NMR1, 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 horn 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, IVa, 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 (Auffray and Rozeon, 1980). PTase 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 94°C and quick chilling on ice, 1 µl of RNasin (40 IU/µl; Promega), 0.1 µl of diithiothreitol, 1.5 µl of dNTP-mix (10 mM of each dNTP; Pharmacia), 6 µl of 5XSuperscript RT buffer, and 1 µl of Superscript (RNase 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 for highly conserved amino acid stretches within the catalytic domains in PTases, 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(A/G/C/T)TA(C/T)TGGCC-3’) was chosen within this 350-bp segment to yield, in combination with primer 2, fragments of this 350-bp segment to yield, in combination with primer 2, fragments of appropriate size for polymerase chain reactions. cDNA, generated by reverse transcription of 2 µg of mRNA isolated from Balb/MK cells, was used as template in a reverse transcription (RT) reaction, and the different 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, Norwalk, 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 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 (Amicon, Beverly, MA). Normalization, according to Patanjali et al (1991), was as follows. A 50–µl reaction mixture containing 0.3 M sodium phosphate (pH 6.8), 0.4 mM EDTA, 0.014% sodium dodecyl sulfate (SDS) and 1 µg of the PTase fragment DNA was overlaid with an equal volume of chloroform and then extracted with a sodium phosphate buffer (pH 7.4). 1 µl RT reaction mix was used for PCR amplification employing primers 2 and 3 as described above. Reassociation of the DNA proceeded for 8 h at 65°C before the addition of 1.0 ml of 0.1 M sodium phosphate (pH 6.8), 0.1% SDS and chilling on ice. The sample was applied to a water-jacketed Bio-Gel HTP hydroxyapatite 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 ethylenediamine tetracetic acid (EDTA)) by Centricron membrane filters (Millipore, MA). TE was washed once with 100 µl of 1 M NH₄Ac. The plasmid DNA (100 ng) was incubated in 500 µl of 0.2 N NaOH for 10 min at room temperature. Following neutralization by addition of an equal volume of 2 M NH₄Ac, 100 µl of denatured plasmid) was loaded per well. After washing the wells twice with 100 µl of 1 M NH₄Ac, the DNA was UV-crosslinked to the membrane (Stratagene, 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 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 prehybridization in 1 M NH₄Ac, 0.1% SDS phosphate buffer (pH 7.4), 1 mM EDTA at 65°C, denature probe was added and membranes were hybridized for 6 h at 65°C. Washing (0.1% SDS, 0.04 M sodium phosphate buffer (pH 7.4), 1 mM EDTA) was performed three times at 65°C for 20 min. Autoradiography was on Kodak X-omat SI films at −70°C for up to 1 day using DuPont Cronex intensifying screens.

**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 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 receptor-type PTases PTPB, LRP, LAR, PTPβ, BPTP1 and PTPε, the cytoplasmic PTP1B, and three novel PTases, 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 (Barnea 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 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, mPTPκ-1, and in particular mPTP9–PTP, 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
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 ‘KCDQYW’P and ‘HCSAVGR’; 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; Barnea et al, 1992; Barnea et al, 1992; Barnea et al, 1992).
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, mPTP14; L, mBPTP1; Epssion, mPTP1; LRP, mLRP-1; vector, plBlue-Script SK+; P19-PTP, mP19PTP; PTP1B, mPTP1B; J3, mPTP13; J4, mPTP38; Beta, mPTP8; GAPDH, rat glyceraldehyde phosphate dehydrogenase cDNA; Gamma, mPTP-1; Kappa, mPTP-1. PTPase cDNA fragments, except mP19PTP, mPTP-1, and mPTP-1c, have been obtained using RNA isolates from five different fractions (I, II, III, IIIa, and IV) of mouse skin cells (Fürstenberger et al, 1986) 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, pBlue-Script SK+ were spotted to reveal background hybridization levels.

REFERENCES


den Hertog J, Pals CEJM, Peppelenbosch MP, Teruel-Lopez GJ, de Laat SW, Kruijver W: Receptor protein-tyrosine phosphatase alpha is activated post-10 days and is involved in neuronal differentiation. EMBO J 13:3789-3798, 1994


Kraus JW, Murphy DP, Carter DM, Krujver JC: Synergistic effects of epithelial growth factor (EGF) and insulin-like growth factor 1 (IGF-I) on keratinocyte proliferation may be modulated by IGF-I transmodulation of the EGF receptor. J Invest Dermatol 96:419-424, 1991


We thank Dr. Stuart Aaronson (The Mount Sinai Hospital, New York) for providing the Balb/MK cells, Paul Mier for stimulating discussions during the preparation of this study, and Dave Iles for critical reading of the manuscript. This work is supported in part by grants from the Dutch Cancer Society (KWF) and the Dutch Organization for Scientific Research (NWO).


Ristow HJ, Messner 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


