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Biochemical and Biophysical Research Communications 325 (2004) 344–352
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α-Hemolysin-induced dephosphorylation of EGF receptor of A431 cells is carried out by rPTPσ

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Received 24 September 2004
Available online 22 October 2004

Abstract

Earlier we have shown that the epidermal growth factor receptor was unable to retain its phospho Tyr signal after the assembly of staphylococcal α-hemolysin (α-HL). However, the nature of the protein tyrosine phosphatase (PTPase) or its identity is not known. In this report, we demonstrate that the α-HL elevates the activity of receptor like protein tyrosine phosphatase σ (rPTPσ). The α-HL induced dephosphorylation is prominent only in intact A431 cells. The PTPase activity is not inhibited if the α-HL treatment precedes PTPase inhibitor treatments. The anti-EGFr immunoprecipitates have exhibited higher PTPase activity after α-HL treatment of A431 cells. Interestingly, PTPase activity of anti-EGFr immunoprecipitates from the A431 cells expressing the antisense message of rPTPσ has not increased despite α-HL treatment, confirming the role of rPTPσ in the dephosphorylation of EGFr. The studies presented here will be useful in understanding the process of signal modulation by the assembly of α-HL.

Keywords: α-Hemolysin; Receptor protein tyrosine phosphatase sigma; PTPase activity; Epidermal growth factor receptor; Dephosphorylation

It is well established that epidermal growth factor receptor (EGFr) communicates to other adapter proteins and downstream signaling molecules through its auto-phosphorylation after stimulation by EGF and TGFα [1,2]. While the events that lead to phosphorylation of EGFr in response to EGF/TGFα are well defined and well understood, the circumstances that lead to its dephosphorylation by the host cell are not completely clear. This intracellular dephosphorylation has to be carried out by specific protein phosphatases. It is very clear from the current understanding that the phosphorylation and dephosphorylation reactions are said to be in a dynamic equilibrium in living cells [3]. The intracellular phosphatases that carry out the dephosphorylation are classified into four distinct classes viz. tyrosine specific, Ser/Thr specific, dual specificity phosphatases, and general phosphatases. The phosphatases that can, in general, control the EGFr could be either tyrosine specific and/or Ser/Thr and/or dual specificity phosphatases as the EGFr is predominantly phosphorylated at the tyrosine residues in response to EGF/TGFα and at Thr654 and Ser1002 residues, which are involved in negative regulation of EGFr. The tyrosine phosphorylation of EGFr in response to EGF/TGFα is extensively mapped but the endogenous PTPases involved in the dephosphorylation of phospho-Tyr (all and/or specific phospho-Tyr) residues are not clear because cells express many protein tyrosine phosphatases (PTPases; [2–4]). In our earlier study, we have suggested that the treatment of A431 cells with α-hemolysin (α-HL), a bacterial pore forming toxin secreted by *Staphylococcus aureus*, led to an activation of a protein tyrosine phosphatase. It was observed that the EGFr was unable to retain its Tyr phosphorylation signal in the presence of an assembled α-HL [5]. We have recently reported that α-HL interacts with functional Caveolin-1 for its assembly on A-431

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doi:10.1016/j.bbrc.2004.10.038

cells. Assembly of α-HL leads to clustering of Caveolin-1 at cell–cell contacts. Hampering the α-HL assembly by either incapacitating the Caveolin-1 or by using Caveolin-1 interacting motif deficient mutant of α-HL prevented α-HL mediated EGFr dephosphorylation [6,7]. These observations suggested a link between α-HL mediated Caveolin-1 reorganization and activation of a PTPase. However, neither the identity of the PTPase nor the mechanism of its activation is clear. In this manuscript, we have presented several evidences about identity of the PTPase involved in the dephosphorylation of EGFr. The phosphatase that selectively dephosphorylates the EGFr, activated by the assembly α-HL, has been identified as the receptor like protein tyrosine phosphatase σ (rPTPσ).

Materials and methods

Anti-phosphotyrosine antibody directly conjugated with horseradish peroxidase (PY20-HRP), anti-epidermal growth factor receptor (anti-EGFr: SC-120 for immunoprecipitation, SC-03 for immunodetection), anti-receptor protein tyrosine phosphatase σ (anti-rPTPσ-N and anti-rPTPσ-C specific for N- and C-termini, respectively), anti-receptor protein tyrosine phosphatase β (anti-rPTPβ), anti-protein tyrosine phosphatase 1B (anti-PTP1B), and corresponding HRP conjugated secondary antibodies were obtained from Santa Cruz Biotechnology, USA. Anti-phosphothreonine and anti-phosphoserine were purchased from Sigma Chemical, USA. Anti-receptor protein tyrosine phosphatase σ rabbit polyclonal serum (anti-rPTPσ-N (322) and anti-rPTPσ-C (320) specific for N- and C-termini, respectively) were a kind gift from Prof. Axel Ullrich (Germany). Non-radioactive PTPase assay kit was purchased from Promega, USA. The A431 cells expressing the rPTPσ-sense, rPTPσ-anti-sense message, and mock transfected cells were a generous gift from Prof. Frank D. Böhmer (Germany) and the cell line was maintained as described by the authors [8]. All other reagents were of analytical grade.

Protein expression in Escherichia coli

Recombinant α-HL was expressed under the control of T7 promoter. The mutants were extensively characterized by the procedures reported earlier [9,10].

Cell culture

The A431 cells were cultured routinely in DMEM supplemented with 5% FCS and antibiotics. The cells at approximately 70–80% confluency were dislodged by either PBS with EDTA (1 mM) or by mild trypsinization (~10 μg/ml trypsin). The integrity of EGFr is not affected by the procedure adopted here [11]. In addition, the integrity of other important cell surface proteins such as EGFr and rPTPσ (data shown in this manuscript) and PLCγ (data not shown) is also not affected as evidenced from the data in this manuscript. The A431 cells expressing the rPTPσ sense and anti-sense message were maintained in DMEM, supplemented with sodium pyruvate, L-glutamine, glucose (4.5 g/l), G418 (0.4 mg/ml), and 10% fetal bovine serum [8].

Influence of α-HL on detergent lysed A431 cell extracts

Approach 1. The A431 cells (~1.5×10^5 cells/reaction) were mock treated or incubated with indicated concentrations of α-HL for 30 min at 37 °C, the unbound toxin was removed, and the cells were lysed in detergent containing buffer (30 mM Hepes, pH 7.4, 10% glycerol, 1% Triton X-100, 1 mM EDTA, and 5 mM EGTA) with or without 50 μM ATP. All the samples were then stimulated with TGFα (70 nM) for 3 min at room temperature. At the end of the incubation, an aliquot of 5× Laemmli sample buffer (LSB) containing protease and phosphatase inhibitors was added and the samples were boiled for 10 min before SDS–PAGE, electrophoretic transfer, and immunodetection.

Approach 2. Detergent lysis of A431 cells was carried out by the lysis buffer (30 mM Hepes, pH 7.4, 10% glycerol, 1% Triton X-100, 1 mM EDTA, and 5 mM EGTA) followed by centrifugation at 10,000*g* for 10 min to remove the debris and the supernatant was used for the experiments. The extracts (5 μg protein/lane) were then treated with the indicated concentration of αHLTGFα for 30 min. At the end of incubation period, samples were transferred to ice and phosphorylation reaction was initiated by addition of 10× reaction buffer (50 mM Hepes, 5 mM $MnCl_2$, 5 μM ATP, 1% Triton X-100, and 100 mM $MgCl_2$). The reaction was stopped after 3 min by the addition of EDTA to 10 mM concentration and 5× Laemmli sample buffer (LSB) and processed for immunodetection.

Immunoprecipitation

The A431 cells (8×10^6–1×10^7) were either mock treated or TGFα stimulated for 10 min at 37 °C (70 nM) or treated with α-HL (200 nM). After all the treatments, the cells were lysed in lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 1 μg/ml leupeptin, 5 mM EGTA, and 2 mM Na_3VO_4), chilled on ice for 20 min, and then centrifuged at 12,000*g* for 10 min at 4 °C. The supernatant was collected and total protein was estimated. About 750–1000 μg of total protein/lane was used for immunoprecipitation (IP). Anti-EGFr (500 ng–1 μg; SC120) was used as primary antibody. After the addition of antibody, the mixture was kept for mild shaking at 4 °C for 4–6 h. The recombinant protein-G agarose bead suspension (30 μl) was then added to the sample and kept for further shaking at 4 °C for 2 h. The beads were then pelleted at 1600*g* at 4 °C and washed with TBS containing 1% Triton X-100, followed by one wash in plain TBS. The final wash and resuspension of the beads was in 50 mM Tris–HCl, pH 7.4, 0.1% Triton X-100. For the detection of rPTPσ, the immunoprecipitates were separated on SDS–PAGE, transferred to nitrocellulose membranes, and probed with appropriate antibodies as described above.

PTPase identification

For the identification of PTPases, the immunoprecipitates obtained by the above procedure were boiled at 95 °C for 10 min in 1× LSB and were transferred to the nitrocellulose membrane using a dot blot apparatus and the membrane was probed with various antibodies as indicated.

Determination of PTPase activity in immunoprecipitates

For PTPase assay, the bead suspension was used directly for in vitro non-radioactive phosphatase assay (Promega, USA) and Na_3VO_4 in lysis buffer was omitted. The beads were suspended in assay buffer (50 mm Tris–HCl, pH 7.4, 0.1% Triton X-100) and the assay was carried out as per the instructions of the manufacturer (Promega). The experiments using the A431 cells expressing the antisense-rPTPσ message were essentially carried out as mentioned above, except that the normal A431 cells were replaced with A431 cells expressing the antisense message of rPTPσ.

Phosphorylation assays with α-HL on A431 cells

Phosphorylation assays were carried out as described earlier [5]. Briefly, the A431 cells (~1×10^5/lane) were stimulated with either

TGFα (70 nM) for 10 min at 37 °C or incubated with the indicated amounts of α-HL at 37 °C for indicated times, following which the toxin was removed by centrifugation. The cells were then stimulated with TGFα and centrifuged. The resultant cell pellets were solubilized in 1× LSB containing 10 μg/ml leupeptin, 2 mM PMSF, and 2 mM Na_3VO_4, denatured for 10 min before subjecting to SDS–PAGE. The proteins were then transferred to nitrocellulose membranes and probed with appropriate antibodies.

Immunofluorescence studies

For immunofluorescence studies, A431 cells (CRL 1555; ATCC) were grown for 48 h on uncoated glass coverslips to different degrees of confluency. Control cells or cells treated with α-HL (200 nM, 15 min) were fixed with 2% formaldehyde freshly prepared from paraformaldehyde in PBS (pH 7.4, 0.12 M sucrose). Autofluorescence was quenched with PBS glycine (100 mM). Unspecific antibody binding was blocked for 1 h with phosphate-buffered gelatine (PBG: PBS, 0.5% bovine serum albumin, and 0.045% cold-water fish gelatine). Primary antibody incubation was done at room temperature for 2 h after dilution in PBG, 1:50 for rabbit antisera 322 (against N-terminus of rPTPσ) or 320 (against C-terminus of rPTPσ) or anti-EGFr antibody (SC-120). After three washes in PBG, primary antibody binding was detected with isotype-specific secondary antibody, Oregon green conjugated Goat-anti-rabbit IgG (1:200) or anti-mouse Texas red. Control coverslips were incubated with secondary antibody alone. Coverslips were mounted using Prolong antifade mounting medium (Molecular Probes) and viewed under laser confocal microscope Ziess LSM 510 under confocal settings.

Quantitation of phosphorylation

All the immunoblots depicting the phosphorylation patterns or the levels of EGFr were routinely quantified by scanning in Bio-Rad phosphorimager software. The TGFα stimulated cells were taken as 100% and the intensities of other bands were expressed as percent of TGFα stimulated lanes.

Results

Rationale

α-HL of *Staphylococcus aureus* is a pore forming toxin that permeabilizes target cell membranes through its assembly, i.e., formation of heptameric ring or pore like assembly. In order to obtain evidence regarding the property of dermonecrosis caused by α-HL, we have investigated the nature of changes that occur in A431 cells well before pore formation. In our earlier experiments, we have been able to obtain evidence regarding activation of PTPase that is responsible for dephosphorylation of EGFr mediated by the assembly (formation of heptameric structures) of α-HL on A431 cells [5]. In this regard, we have ruled out several obvious possibilities such as interference in receptor dimerization, loss of ATP, receptor shedding, receptor down-regulation, and role of negative regulatory elements, which otherwise would have affected the phosphorylation status of EGFr. These experiments in essence conveyed the message that the assembly of α-HL is vital for observing the enhanced PTPase activity. The increase in putative PTPase activity was not observed in the presence of an α-HL mutant deficient in oligomeric assembly. Moreover, the EGFr of stimulated A431 cells also lost its existing phosphorylation when α-HL was allowed to assemble on pre-stimulated cells, confirming the role of a PTPase [5]. Here, we have presented several evidences regarding the identification of the PTPase that is involved in the dephosphorylation of EGFr.

α-HL mediated dephosphorylation requires intact cells

It is clear according to the literature that the dephosphorylation of cellular targets is being carried out by cellular phosphatases and in this particular instance, i.e., dephosphorylation of EGFr of A431 cells, it is a protein tyrosine phosphatase. While the phosphatases are distributed in various compartments of a cell, a particular phosphatase is presumed to target its substrate in response to a given condition/requirement [1,3,4,12,13]. In view of this, we addressed the question as to whether α-HL requires an intact cellular environment for the dephosphorylation or α-HL can also induce the dephosphorylation of EGFr in detergent lysed A431 cells, in which the native cellular architecture is totally disrupted. Under such conditions the recruitment, if any, of the cell surface molecule(s) for the induction of dephosphorylation is either difficult or does not occur. To this end, we chose two approaches. In the first, intact cells were initially treated with the α-HL for the required length of time and lysed in detergent containing buffer (see Materials and methods) before stimulation with TGFα. In the second approach, the A431 cell lysate was prepared prior to the treatment with α-HL. In view of the possibility that α-HL is easily neutralized by Triton X-100 (used to solubilize the EGFr), it is difficult to judge the interaction between α-HL and EGFr. To obviate this problem, we have physically fused α-HL to TGFα as it is easy to evaluate the function of TGFα. It is also well known that TGFα stimulates the Triton X-100 solubilized EGFr. The data shown in Fig. 1 suggest the requirement of intact cells for the α-HL induced dephosphorylation of EGFr as the α-HL could not induce the dephosphorylation of EGFr present in the detergent lysed A431 cell extracts. This experiment also shows that the α-HL is in close proximity to the EGFr and yet unable to cause the dephosphorylation. Hence, interaction of α-HL with EGFr is not responsible for the observed dephosphorylation. The latter observation is also consistent with an earlier report in which it was found that the phosphorylation status of EGFr present in PC12 cell extract is not affected by the addition of α-HL [14]. These data shown in Fig. 1A once again reiterated our earlier observation that the dephosphorylation of EGFr was due to a hyperactive PTPase rather than the possibility of loss of ATP or a direct interaction between α-HL and EGFr. This conclusion

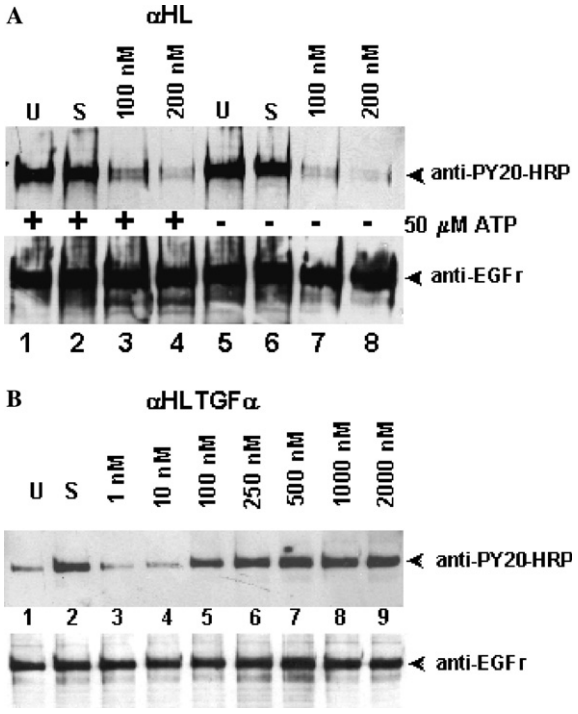

Fig. 1. Effect of α-HL/αHLTGFα on EGFr tyrosine phosphorylation in (A) intact (B) detergent lysed A431 cells. (A) The A431 cells were incubated with indicated concentration of α-HL (lanes 3, 4, 7, and 8) for 30 min at 37 °C, the unbound toxin was removed, and the cells were lysed in detergent containing buffer. The rest of the experiment is carried out as described in Materials and methods. Upper panel represents the phosphorylation status of EGFr obtained by probing with HRP-conjugated anti-phosphotyrosine antibody (anti-PY20-HRP). Lower panel was obtained by reprobing the above blot with anti-EGFr antibody after stripping the anti-PY20-HRP. (B) Detergent lysed A431 cell lysate was prepared as described in Materials and methods under Approach 2 and treated with the indicated concentrations of αHLTGFα. The remainder of the experiment is carried out as described in Materials and methods. Upper panel and lower panels were obtained as in (A).

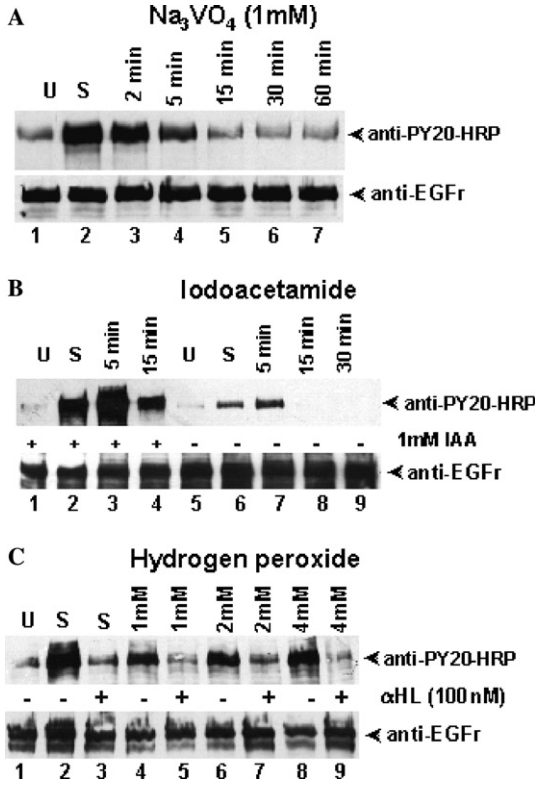

Fig. 2. Effect of α-HL on EGFr phosphorylation in the presence of: (A) Na_3VO_4, the A431 cells (1.5×10^5 cells/lane) were pretreated with 1 mM Na_3VO_4 and incubated with α-HL for indicated times. The rest of the procedure is same as described in Materials and methods (see under phosphorylation assays). The untreated and TGFα (70 nM) stimulated cells in the presence of Na_3VO_4 are shown in lanes 1 and 2, respectively. (B) Iodoacetamide (IAA), the A431 cells (1.5×10^5/lane) were treated with IAA (1 mM) in PBS, pH 7.7, at RT for 30 min and subjected to α-HL treatment. (C) Hydrogen peroxide, the A431 cells were incubated with α-HL (100 nM, lanes 3, 5, 7, and 9) for 30 min at 37 °C or mock treated (lanes 2, 4, 6, and 8) under similar conditions, followed by toxin removal. Cells were then treated with indicated concentrations of H_2O_2 (lanes 4–9). The U and S, respectively, represent untreated cells and cells stimulated with TGFα (lanes 1 and 2). Lane 3 cells pretreated with α-HL for 30 min, followed by stimulation with TGFα for 10 min.

is also consistent with our earlier observation in which the presence of α-HL was not observed in the immunoprecipitates obtained with anti-EGFr antibody after α-HL treatment [6].

Effect of PTPase inhibitors on α-HL mediated dephosphorylation

The active site cysteine of PTPases (H**C**XXGXX**R**(S/T)) can be modified by a variety of alkylating reagents, oxidants, etc. PTPases can also be inhibited with the well-known inhibitor sodium orthovanadate (Na_3VO_4) [3,15,16]. Inactivation of cellular PTPases (either with modifying agents or Na_3VO_4) shifts the dynamic equilibrium of the phosphorylation–dephosphorylation reactions towards the phosphorylation of RTKs (referred to as ligand independent phosphorylation). In the present study we have employed three different inhibitors as described in Fig. 2 viz. (1) Na_3VO_4, which mimics the transition state of catalysis. (2) Iodoacetamide, an irreversible alkylating agent of the active site Cys of PTPases. (3) Hydrogen peroxide, a reversible inhibitor of the active site Cys of PTPase.

We have noticed a distinct difference in the final status of EGFr phosphorylation depending on the sequence of treatment, i.e., if the cells are treated with α-HL followed by an inhibitor like Na_3VO_4 then the Na_3VO_4 is practically ineffective in inhibiting the PTPase, which is consistent with our earlier report [5]. This could be due to the fact that once a PTPase is committed for dephosphorylation, its active site confirmation is altered due to the formation of thiophosphate linkage and at that stage, the Na_3VO_4 cannot compete or displace the bound phosphotyrosine making it an ineffective inhibitor. If the cells are treated with Na_3VO_4

first followed by treatment with α-HL, the induction of dephosphorylation was retarded, i.e., it takes more time for the α-HL to induce dephosphorylation (Fig. 2A). The marginally long lag time could be due to three specific reasons. (1) The assembly of α-HL is retarded because assembly is necessary for inducing the dephosphorylation. (2) Generation of active PTPase i.e., displacement of the Na_3VO_4 complexed with the PTPase may take long time. (3) A tiny fraction of PTPase is not inhibited by $Na_3VO_4$and can be utilized by α-HL for its action. Based on our experiments we have already proved that the α-HL assembly was practically complete by 5–15 min after its addition to the cells, hence, the first reason was ruled out. However, it is well known in the literature that the Na_3VO_4 is quickly reduced once inside the cell making it a poorer inhibitor in comparison to Na_3VO_4. It has been demonstrated that $Vanadate^{(+5)}$ is reduced to $Vanadyl^{(+4)}$ once it enters the cells [17–19]. Importantly, $Vanadate^{(+5)}$ has been shown to be more efficacious as PTPase inhibitor than $Vanadyl^{(+4)}$. Although, the remaining active fraction of PTPase, if any, after treatment with Na_3VO_4 might be sufficient to induce the dephosphorylation, especially due to their extremely short $t_{1/2}$ times [20–22].

In view of partial inactivation of Na_3VO_4 inside the cellular environment, we have employed an irreversible inhibitor, i.e., iodoacetamide, which is a potent alkylating agent of Cys residues of proteins. The data shown in Fig. 2B clearly illustrate that the PTPase was reasonably inactivated and as a result, the EGFr was able to retain the signal despite α-HL treatment.

The third is ligand independent activation of EGFr by hydrogen peroxide. Hydrogen peroxide shifts the equilibrium of phosphorylation–dephosphorylation reactions towards phosphorylation by inhibiting the reverse reaction. Hence, we examined as to how α-HL influences the PTPase activity modulated by hydrogen peroxide. Please note that in these experiments, the cells were treated with α-HL first followed by treatment with hydrogen peroxide. As expected, the α-HL treated cells clearly exhibited an active PTPase as shown in Fig. 2C. The explanation for the inability of the hydrogen peroxide to inhibit the PTPase is the same as explained above, i.e., once the PTPase committed for dephosphorylation is not inhibited by Na_3VO_4 or iodoacetamide or hydrogen peroxide essentially because the active site Cys is covalently linked with the phosphate, i.e., formation of thiophosphate linkage.

A feature common to all the three inhibitors was that the PTPase activity was not 100% suppressible as the EGFr phosphorylation has appeared to diminish with time, i.e., >90 min range after α-HL treatment. We believe that there may be a tiny fraction (may be about 5–10%) of PTPases in an oxidized state. This observation is in agreement with the data discussed by Suo Gho Rhee's group, who reported that about 6% of PTPases were not inactivable with [^{14}C]iodoacetic acid [16]. It is not yet clear whether this fraction gains activity after the assembly of α-HL. In summary, the PTPase inhibitors are effective only when they arrive prior to the arrival of α-HL but not the reverse. It is reasonable to conclude that the α-HL induced dephosphorylation is a context based (membrane perturbation) activation of a PTPase.

Identification of PTPase and its activity

In the light of these observations, we have attempted to identify the PTPase responsible for the dephosphorylation of EGFr by following a similar approach described by Wang et al. [23] and Wu et al. [24]. These authors have identified the SHP-2 as a dual specificity phosphatase which dephosphorylates STAT1 using an antibody array (dot blot) approach. In order to identify the PTPase involved in the process, we have immunoprecipitated the detergent lysed cell extracts of untreated, TGFα stimulated, and α-HL treated cells with anti-EGFr antibody and the resultant proteins were screened by employing an array of antibodies. By using this procedure, we have identified the phosphatase responsible for this irreversible dephosphorylation of EGFr as the receptor like protein tyrosine phosphatase σ (Fig. 3A). We have obtained an unambiguous signal with both anti-rPTPσ-N and anti-rPTPσ-C antibodies (specific for intact N- and C-termini of rPTPσ, respectively). Under identical conditions, neither the HRP conjugated secondary antibody alone (in the absence of primary antibody) nor did the primary antibody in the presence of the peptide used for its generation give any signal. We have further authenticated the anti-rPTPσ-N antibody for its ability to recognize the N-terminal 371 amino acid fragment of rPTPσ (data not shown). All these observations lend support to our identification approach. To the best of our knowledge, this is the first instance where the endogenous rPTPσ has been shown to be responsible for the dephosphorylation of EGFr mediated by the assembly of α-HL on A431 cells. It may be relevant to mention here that the EGFr as well as its downstream molecule, PLCγ, were also found to be dephosphorylated by the assembly of α-HL on A431 cells (data not shown). It is interesting to note that both rPTPβ and PTP1B are not found in the dot blot approach adapted by us.

We then investigated for the net PTPase activity of immunoprecipitates obtained with the anti-EGFr antibody of untreated, TGFα stimulated, and α-HL treated A431 cell lysates. The total phosphatase activity observed in the immunoprecipitates obtained with anti-EGFr antibody was twofold higher in α-HL treated cells when compared to both untreated and TGFα stimulated cells (Fig. 3B). While it can be argued that, a cell may have many PTPases is the observed increase in total

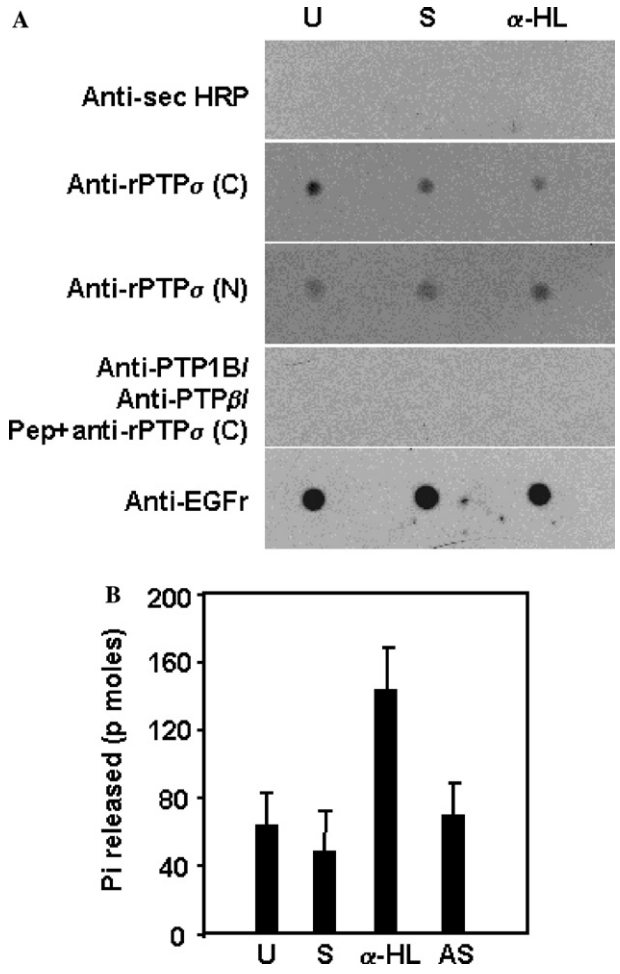

Fig. 3. (A) Detection of PTPases. The mock treated (U), TGFα stimulated (S), and α-HL treated A431 cells (α-HL) were immunoprecipitated with anti-EGFr antibody and blotted on nitrocellulose sheets as described in Materials and methods. The same blot was stripped and reprobed with the indicated antibodies. The anti-EGFr panel confirms the equal loading as determined by densitometric scanning. Please note that the antibody concentrations, blocking, probing, and exposure to the film and film developing conditions were identical in all the cases. (B) Tyrosine phosphatase assay of the immunoprecipitates. The immunoprecipitates were obtained as described above and the tyrosine phosphatase assay was carried out as per manufacturer's instructions (non-radioactive tyrosine phosphatase assay system, Promega, USA). The phosphate released (picomoles) by these samples was calculated from the standard curve. AS represents the amount PTPase activity observed in the α-HL treated immunoprecipitate of A431 cells expressing the antisense-rPTPσ message. The activity data are an average of five independent immunoprecipitation experiments.

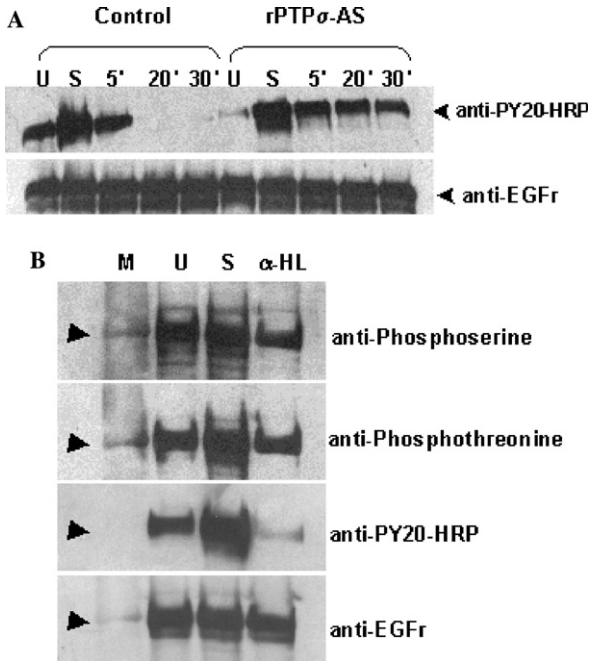

Fig. 4. (A) Effect of α-HL treatment on phosphorylation status of EGFr in rPTPσ anti-sense A431 cell line. Phosphorylation assays of A431 cells transfected with vector alone (control) and rPTPσ anti-sense stable clones were carried out after serum starvation for 6–8 h at 4 °C as reported earlier [5]. Lanes marked with U and S are mock treated with PBS and stimulated with TGFα (70 nM for 10 min at 37 °C), respectively. α-HL treatment was with 200 nM for indicated periods of time, i.e., 5, 20, and 30 min. α-HL treatment was followed by stimulation with TGFα (70 nM) for 10 min, at 37 °C. Upper and lower panels were obtained as described in Fig. 1. (B) Effect of α-HL on Ser-Thr-specific phosphatases. The mock treated with PBS (U), TGFα stimulated (70 nM) for 10 min at 37 °C (S) and α-HL treated A431 cells (100 nM for 30 min at 37 °C) were lysed and immunoprecipitated with anti-EGFr antibody as described in Materials and methods and the immuno precipitates were transferred to nitrocellulose sheets after electrophoresis. The membrane was blocked and probed with anti-EGFr, anti-phosphotyrosine, anti-phosphoserine, and anti-phosphothreonine antibodies successively. The stripped blots were examined for background before reprobing.

PTPase activity of α-HL treated cells per se due to rPTPσ? In order to ascertain the involvement of rPTPσ, we have used the A431 cells expressing the antisense message of rPTPσ [8]. It is clear from Fig. 3B that the net PTPase activity of α-HL treated, antisense-rPTPσ cell line is same as that of basal level, indicating that the observed increase in PTPase activity is predominantly contributed by rPTPσ. In addition, it is clear from Fig. 4A that the EGFr of antisense-rPTPσ cell line is able to retain the phosphorylation signal for longer periods of time, despite α-HL treatment (compare the intensities of 30 min time points in Fig. 4A). We could not achieve 100% suppression of rPTPσ with the anti-sense message owing to the limitations of the approach. Unfortunately, the siRNA approach to suppress the rPTPσ has not yielded consistent results. As a result the residual endogenous rPTPσ is highly efficient and enough to influence the dephosphorylation of EGFr. This is also consistent with the published observations of Frank Bohmer and colleagues. For example, these authors observed that excess GM3 (which modulates the signal of EGFr with the help of rPTPσ) did not reduce the phosphorylation signal of EGFr completely, even in the presence of over expressed rPTPσ [8]. It has been suspected by the authors that the endogenous levels of rPTPσ may alone be sufficient to modulate the activity of EGFr. Our data support this observation as the endogenous rPTPσ has displayed a significant

activity and it explains the logic behind their extremely low endogenous concentrations. It is interesting to note that TGFα stimulated cells exhibited slightly lower PTPase activity which is in excellent agreement with the observation that growth factor stimulation reduces the phosphatase activity due to the production of intracellular H_2O_2 that reversibly inactivates the PTPases in vivo [16,25–27]. In addition, we have not observed any changes in the level of Ser-Thr-phosphorylation pattern of EGFr upon α-HL treatment (Fig. 4B). This observation rules out the role of Ser-Thr- and dual specificity phosphatases in the α-HL induced dephosphorylation. Hence, the elevated PTPase activity observed in the α-HL treated cells is contributed by the rPTPσ (present on the cell membranes) but not by PTP1B (present in the cytoplasmic side of ER) or rPTPβ (or another cell surface PTPase).

rPTPσ processing and immunofluorescence studies

Biochemical and confocal studies carried out by Axel Ullrich and colleagues [28] showed that the rPTPσ undergoes proteolytic processing in response to changes in the levels of Ca^{2+} induced by Ca^{2+} specific ionophore A23187 or phorbol ester TPA treatment in A431 cells. According to the authors, after processing, the extracellular domain of rPTPσ was shed, and thus it is no longer detectable with anti-rPTPσ (N) antibody. The transmembrane and intracellular domains of the remaining rPTPσ were found to be internalized and redistributed away from plasma membrane, as visualized by anti-rPTPσ C-terminal antibody. Calcium withdrawal also led to the internalization of full length rPTPσ without any processing. Keeping these observations in mind, the present study was carried out to examine whether the rPTPσ undergoes any processing and redistribution upon α-HL treatment of A431 cells. Knowing that the α-HL is a pore forming toxin capable of mobilizing Ca^{2+} from extracellular region through its pore (very much like an ionophore), we examined whether the rPTPσ has any change in its net PTPase activity and/or undergoes proteolytic processing. The data in Fig. 5A convey

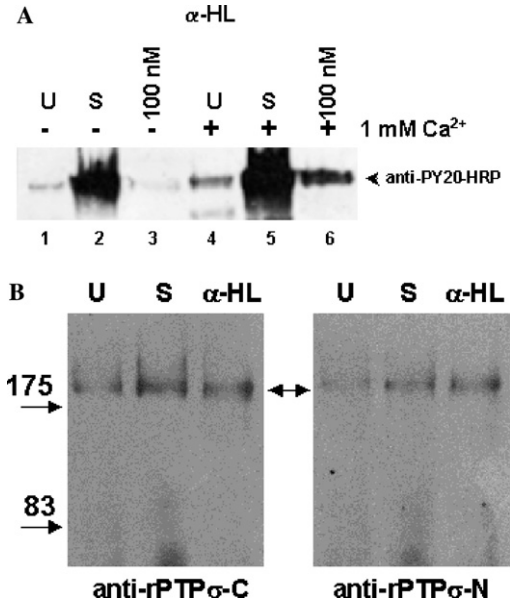

Fig. 5. (A) The influence of Ca^{+2} ion. The effect of α-HL (100 nM) in the presence and absence of Ca^{+2} (1 mM) on the dephosphorylation of EGFr. The experiment was carried out as described in Fig. 1A, except for the presence or absence of calcium. The panel represents the phosphorylation status of EGFr obtained with anti-PY20-HRP. The '+' and '−' represent the presence and absence of calcium. (B) Absence of processing of rPTPσ. The immunoprecipitates with anti-EGFr antibody were obtained as described in Fig. 3A, except that here we have used rPTPσ over-expressing cell line instead of normal A431 cells. The immunoprecipitates were subjected to SDS–PAGE and then transferred onto nitrocellulose membrane, blocked and probed with anti-rPTPσ N- and C-terminal specific antibodies. Full-length 212-kDa rPTPσ protein is easily detectable. Lanes designated with U, S, and α-HL, respectively, represent untreated (or mock treated), TGFα and α-HL treated samples. These data indicate the absence of processing of rPTPσ even after α-HL treatment.

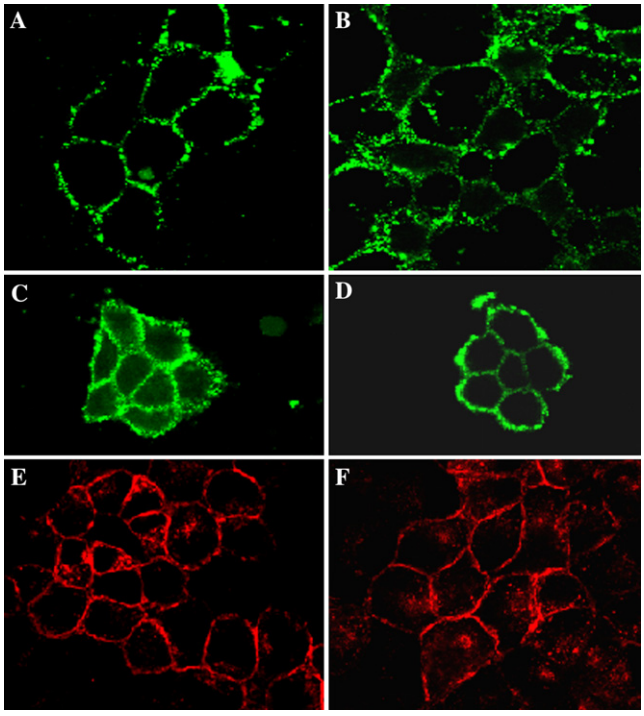

Fig. 6. Immunofluorescence studies of rPTPσ on α-HL treated A431 cells: A431 cells were grown on glass coverslips and kept untreated or treated with α-HL at a final concentration of 200 nM. (A) Immunostaining of rPTPσ was carried out with anti-rPTPσ N-terminal specific antibody (322), which shows an even membrane localization of rPTPσ in untreated cells. (B) Staining with rPTPσ N-terminal antibody after α-HL treatment. No significant difference is observed in the distribution of rPTPσ. (C) Immunofluorescence image of untreated A431 cells stained with anti-rPTPσ-C specific antibody (320) showing even membrane distribution. (D) The immunostaining pattern of α-HL treated A431 cells obtained by staining with anti-rPTPσ-C antibody after α-HL treatment that shows no change in the distribution of rPTPσ. (E) EGFr distribution in untreated A-431 cell surface. (F) EGFr distribution after α-HL treatment remains unchanged.

to us that the rPTPσ activity has not changed, i.e., neither enhanced nor decreased due to the presence of additional calcium or absence of calcium. Regarding proteolytic processing, we should have seen smaller fragments detectable by the N- and C-terminal specific antibodies of rPTPσ in Fig. 5B, if it had occurred. The absence of smaller fragments and lack of noticeable changes in the net PTPase activity in the absence of calcium led us to conclude that the rPTPσ does not undergo any proteolytic processing due to the α-HL assembly.

It is clear from Fig. 6 that rPTPσ distribution remains unchanged after α-HL treatment. Both N- and C-terminal antibody staining shows surface localization of rPTPσ in control as well as α-HL treated cells. No processing/shedding or redistribution of rPTPσ is triggered upon α-HL treatment. This is consistent with the observation that α-HL did not affect the distribution of EGFr (Fig. 6F and [7]). Since rPTPσ is associated with EGFr as shown in Fig. 5B, its distribution was expected as that of EGFr on A-431 cell surface. This leads to the speculation that the activity of rPTPσ is independent of its processing or redistribution and might be just due to disengagement from Caveolin-1.

Discussion

It has been reported earlier that in case of fibroblasts, mild treatment with trypsin or PBS results in a dose and time dependent loss of phosphorylation of cellular proteins, mainly at focal contact proteins [29]. This led to cell rounding and detachment from the substratum. The loss in phosphotyrosine was attributed to the activation of a protein tyrosine phosphatase. Inhibition of the PTPase with phenylarsine oxide or sodium orthovanadate plus H_2O_2 led to retention of tyrosine phosphorylation upon trypsin treatment as well as no cell rounding was observed. Consistent with these findings, treatment of A431 cells with α-HL has resulted in loss of tyrosine phosphorylation of cellular proteins, especially the EGFr [5] and PLCγ (unpublished data). This might also explain the rounding of A431 cells upon α-HL treatment within minutes. While trypsinized cells, upon plating back in growth medium, adhere and restore the tyrosine phosphorylation of focal contact proteins [29], α-HL treated cells do not adhere to the substratum (data not shown), probably due to an irreversible activation of rPTPσ.

Furthermore, it was recently reported that several PTPases of A-431 cells target, in part, lipid rafts and were shown to interact with the scaffolding domain of Caveolin-1 by immunoprecipitation. Among the PTPases, only 10% of the total PTP1B was localized in lipid raft fraction in comparison to 60% of LAR, a receptor like PTPase, which has high homology to rPTPσ [30]. This observation is consistent with our dot-blot data, which did not exhibit the presence of PTP1B in anti-EGFr immunoprecipitates (Fig. 3A). It has been shown earlier that the PTP1B is predominantly located on the cytoplasmic side of the endoplasmic reticulum and complexes with EGFr only upon its internalization [31]. It is relevant to mention here that the α-HL treatment of A431 cells did not lead to internalization or down regulation of EGFr because down regulation does not occur within minutes [5]. We have recently reported that α-HL interacts with Caveolin-1 for its assembly through protein–protein interactions and leads to its clustering at cell–cell contacts while the EGFr remained evenly distributed at the periphery of the cell [6,7]. It is clear from Fig. 6 that the α-HL treatment did not change the overall distribution of rPTPσ on A-431 cell surface much like the EGFr distribution [7]. It is worth mentioning here that rPTPσ remains associated with EGFr at all times as shown in Fig. 5B and any change in distribution of one is anticipated to reflect in the other molecule. Based on these observations, the activation of rPTPσ can be explained on the following possibility: rPTPσ has high sequence similarity with LAR and like LAR has putative Caveolin-1 interacting motif viz. ^{1807}**VRQFQFTDW**^{1815}. Hence, it is possible that rPTPσ remains in an inactive state when associated with Caveolin-1. After α-HL establishes contact with the Caveolin-1 for its assembly, the rPTPσ might be disengaged from the negative regulation of Caveolin-1 scaffolding domain, leading to its activation. This possibility is consistent with the observation that assembly of α-HL is a prerequisite for activation of PTPase [5,7].

In summary, our observations suggest that the α-HL might be exploiting a regulatory mechanism present in the cells and can dephosphorylate all (tyrosine) phosphorylation signals, irrespective of the state of the cell surface receptors. Our data attempt to change the existing paradigm that pore forming toxins simply damage target cell membranes. In addition to membrane damaging property, they have an exquisite ability to modulate signal transduction events without entering the cells.

Acknowledgments

The authors thank Prof. Frank D. Bõhmer for providing the A431 cell line expressing the antisense-rPTPσ message and Prof. Axel Ullrich for providing rPTPσ specific antisera. The authors thank Prof. Surolia, Prof. Padmanaban, and Dr. Mishra for valuable criticism, Dr. Vandana and Dr. Navneet helpful discussions, and Mr. Anil Lotke and Ms. Ashwini Atre for technical assistance. S.K. and R.V. are recipients of senior research fellowship from CSIR. The financial assistance was provided by the Department of Biotechnology, Government of India.

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