Modulation of EGF receptor autophosphorylation by α-hemolysin of *Staphylococcus aureus* via protein tyrosine phosphatase

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Abstract In the presence of assembled α-hemolysin (α-HL) of *Staphylococcus aureus*, the epidermal growth factor receptor (EGFr) is rapidly dephosphorylated. Several obvious possibilities that otherwise would have contributed to the dephosphorylation were ruled out. Instead, an elevation in the activity of a protein tyrosine phosphatase appears to be responsible for the observed loss of phosphorylation signal of EGFr. For this dephosphorylation, the assembly of α-HL is necessary while lytic pore formation is not required. In summary, the EGFr is unable to retain its phosphorylation signal in the presence of α-HL and the process is irreversible.

Key words: α-Hemolysin; Epidermal growth factor receptor; Pore formation; Tyrosine phosphorylation; Dephosphorylation

1. Introduction

The transmembrane pores formed by α-hemolysin (α-HL) of *Staphylococcus aureus* trigger several pathophysiologically important secondary cellular reactions [1–4]. In essence, the action of this toxin on nucleated cells may be diverse and more complex than on red blood cells. In case of susceptible cells, the α-HL has been shown to insert the amino acid region 118–140 into the membrane bilayer to form lytic oligomer, whereas resistant cells can prevent such an insertion [5]. Despite these insights, the mechanism by which α-HL acts on nucleated cells for example on dermal cells is not clear (α-HL has been attributed dermonecrotic property). In other words, what are the events that occur immediately after the binding of α-HL? Can α-HL disrupt the signal transduction cascades of the cell? It is generally assumed that α-HL being a pore forming toxin, can simply deplete a cell of its essential ingredients leading to its death. Lazarovici and Chan had observed, in PC12 cells upon treatment with α-HL, a decrease in the affinity of the epidermal growth factor receptor (EGFr) for epidermal growth factor [6]. The pathway of action of α-HL has remained, however, unexplained. Considering the involvement of *S. aureus* in pathogenesis in humans, it is essential to carry out an in depth study in order to understand the early events that occur when α-HL acts on EGFr containing cells. Such a study can help us understand the molecules that mediate or act in synergy to regulate the EGFr function [7–10]. With this aim, we have investigated the mechanism of action of α-HL based on its structure, assembly and function using the A431 epidermoid carcinoma cell line [21]. Our data provides an important clue regarding the role of a protein tyrosine phosphatase (PTPase) which can dephosphorylate the EGFr in the presence of α-HL.

2. Materials and methods

All the reagents used were of analytical grade and all experiments described here were carried out independently at least three times. Transforming growth factor α (TGFα) and in vitro transcription and translation kit were obtained from Promega, Madison, WI, USA. Fetal calf serum (FCS) was purchased from Harlan, Sera Lab. RPMI, anti-phosphotyrosine antibody 6G9 and anti-EGF receptor antibody (sheep polyclonal) were obtained from Gibco BRL. Horseradish peroxidase (HRP) conjugated anti-phosphotyrosine antibody (PY20–HRP) and anti-EGFr antibodies (SC-120 and SC-03 were respectively used for immuno precipitation and immunodetection) were from Santa Cruz Biotech, Santa Cruz, CA, USA. HRP conjugated anti-mouse antibody and chemiluminescence detection kit were purchased from New England Bio-Labs.

2.1. Purification of α-HL, α-HL(5–293) and H35N

The mature form of α-HL is a 293 amino acid long (A^1-D-S-D-I...E-E-M-T-N^{293}) protein [11]. α-HL(5–293) is an amino terminal deletion mutant of α-HL lacking the first four amino acids (A^1-D-S-D^4) which assembles into the heptameric pores like α-HL [11]. The H35N mutant of α-HL (histidine-35 of α-HL is replaced with aspargine) was constructed as described earlier [12]. α-HL, α-HL(5–293) and H35N were cloned and expressed in *E. coli* (JM109(DE3) or BL21(DE3)) under control of T7 promoter. [^{35}S]Methionine labeling of α-HL, α-HL(5–293) and H35N was achieved by coupled in vitro transcription and translation using the super coiled plasmids as described earlier [11–13].

2.2. Cell culture

A431 cells were cultured in RPMI medium buffered with 2.5 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate (HEPES) supplemented with 5% FCS and antibiotics (streptomycin sulfate and penicillin G). Cells at approximately 70–80% confluency were trypsinized with 10–50 μg/ml trypsin. The cells were starved in a serum free medium for a minimum of 10–12 h before the experiment. We have not observed any degraded EGFr fragments of low molecular weight which could have arisen due to the mild trypsinization, as judged by immunodetection of EGFr. This is consistent with the observations of Chinkers and Brugge as the extracellular portion of EGFr has no susceptible loci for trypsin [14]. In addition, experiments were also performed by dislodging the monolayer with phosphate buffered saline (PBS) containing 1 mM EDTA (without trypsin) and the dephosphorylation effect induced by α-HL was consistently observed.

2.3. Binding and oligomerization on A431 cells

The A431 cells were incubated with 3 μl of the [^{35}S]methionine

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labeled α-HL or α-HL(5–293) (3 μl) or H35N (12 μl), in a total volume of 33 μl at 37°C. Aliquots of cells (11 μl; 4×10^4) were taken out at 2, 5 and 15 min and the cell pellet was washed once with Dulbecco's PBS (D-PBS). This was followed by addition of 1×Laemmli's sample buffer (LSB; 25 μl). The samples were heated at 60°C for 10 min and electrophoresed on 9% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli [15]. The resultant gel was dried and kept for autoradiography.

2.4. Assay for phosphorylation of EGFr using anti-phosphotyrosine antibody

A431 cells (2.0×10^5) were incubated with an indicated amount of the toxin in a total volume of 50 μl at 37°C for indicated time periods. The cells were washed once with D-PBS to remove the unbound toxin and finally resuspended in 50 μl of D-PBS. The cells were stimulated at 37°C for 10 min with TGFα (20 ng; 66 nM). The cell pellet was recovered by centrifugation, resuspended in LSB (20 μl) containing protease inhibitors (10 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM Na_3VO_4, boiled for 10 min and electrophoresed on 7% SDS–PAGE after centrifugation. The proteins were transferred to a nitrocellulose membrane using 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid buffer (pH 10.5) at 250 mA for 2 h. The blot was subjected to immuno detection with anti-phosphotyrosine antibody. The cells treated with TGFα exhibited a slightly diffuse EGFr band as a result of its phosphorylation [16]. For the experiments in the presence of tyrosine phosphatase inhibitors, an identical protocol was followed with the exception that the A431 cells (2.0×10^5) were preincubated in D-PBS containing 50 μM Na_3VO_4 and 50 μM NaF at 37°C for 30 min and the phosphatase inhibitors were maintained throughout the course of experiment. In the experiments with prestimulated cells, the cells were first treated with 66 nM TGFα followed by treatment with α-HL. The rest of the protocol was the same as described above. Binding, oligomerization and phosphorylation assays on other cells lines were essentially similar to those carried out on A431 cells.

2.5. Quantitation of phosphorylation

The bands obtained after immunodetection with anti-phosphotyrosine antibody or with anti-EGFr antibody were quantified by scanning using a Bio-Rad phosphor-imager software. The band intensity of TGFα stimulated cells was taken as 100% and the intensity of other bands was expressed as percent of TGFα stimulated lanes.

2.6. ATP release assays

The ATP released in response to the binding of α-HL was assayed by ATP Bioluminescence kit (Boehringer-Mannheim). For these experiments, 3×10^5 cells were suspended in medium without FCS and were incubated with or without α-HL (50 nM) for different time periods. The cells were pelleted and the amount of ATP released into the medium was measured by adding 50 μl of the bioluminescence reagent to 50 μl supernatant. The light intensity was measured by using a Perkin-Elmer LS 50B spectrofluorimeter operating in luminescence mode at 550 nm. For measuring the intracellular ATP, the cells were lysed with 50 μl of 0.5% Triton X-100, centrifuged at $10\,000\times g$ for 1 min and the ATP content present in the supernatant was measured.

2.7. Assay for receptor dimers

The A431 cells (1×10^6) were incubated with indicated amount of α-HL for 30 min at 37°C in a total volume of 50 μl. The cells were washed, resuspended in 46 μl of D-PBS, stimulated with 40 ng (4 μl, 130 nM) TGFα followed by incubation at 37°C for 30 min. The volume was made up to 190 μl with D-PBS. Glutaraldehyde (10 μl) was added to a final concentration of 500 μM. The cross-linking reaction was allowed to proceed for 1 min at room temperature and the reaction was quenched by the addition of glycine (pH 9.0) to a final concentration of 5 mM. After an additional 10 min, the cells were centrifuged and lysed in lysis buffer (50 mM HEPES, 0.5% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1.0 mM EDTA, 3.0 mM $MnCl_2$, 2.0 mM Na_3VO_4, 10 μg/ml leupeptin, 2 mM PMSF) on ice for 1 h. The lysate was clarified at 10 000 rpm for 10 min and supernatant was precipitated with 8 volumes of prechilled acetone at −70°C. The precipitate was collected by centrifugation, dried and solubilized in 1×LSB, boiled for 10 min, and electrophoresed on 5% SDS–PAGE. The proteins were transferred onto nitrocellulose membrane at 250 mA for 2.5 h as described above. The blot was probed with an anti-phosphotyrosine antibody.

3. Results and discussion

Among the toxins secreted by *S. aureus*, α-HL is a 293 amino acid long monomer which has excellent solubility in extracellular medium. This monomeric form, also known as the 'water soluble' form, efficiently assembles into heptameric pores on target cell membranes. During this pore formation, the water soluble monomeric α-HL (stage 1) binds to target cell membranes as a monomer (or protomer). This cell bound monomer (stage 2) interacts with other such monomers to form a non-lytic, pre-pore (stage 3). This pre-pore undergoes further conformational changes to form a lytic pore or transmembrane channel (stage 4). The carboxy terminal deletion mutants of α-HL (deletion of two to four amino acids) are unable to fold properly and hence lack efficient inter protomer interactions to form the non-lytic, pre-pore (i.e. are unable to assemble beyond stage 2). In contrast, the amino terminal deletion mutants (two to four amino acid deletions) were found to be arrested at stage 3 as non-lytic pre-pores [11–13,17]. Only the full length α-HL is able to form the lytic pore efficiently (stage 4). In addition, the histidine-35 of α-HL is essential for oligomerization of the toxin. Although H35N folds much like the water soluble form of α-HL, it

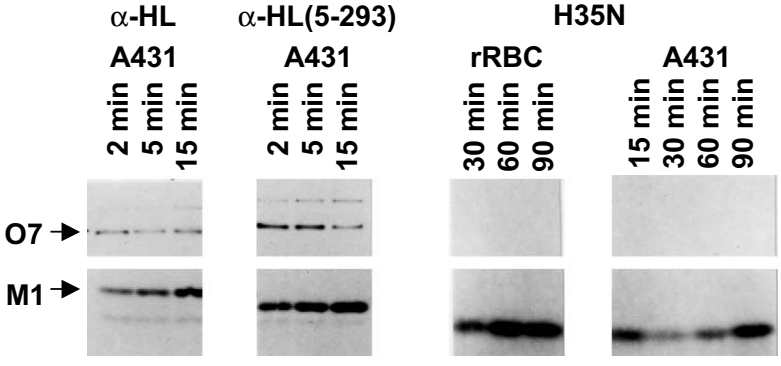

Fig. 1. Binding and oligomerization of α-HL, α-HL(5–293) and H35N on A431 cells: [^{35}S]Met labeled α-HL, α-HL(5–293) and H35N were incubated with A431 cells, the unbound toxin was removed by centrifugation and the cell lysate was subjected to 7% SDS–PAGE. Four fold higher amounts of [^{35}S]methionine labeled H35N was employed to detect even the weaker oligomeric band of H35N, if at all present. The bands O7 and M1 represent the heptameric oligomer (~200 kDa) and respective monomers of α-HL (33 kDa). The oligomer bands (O7) were further authenticated by reelectrophoresis after extraction from unfixed, dried gels as described earlier [11].

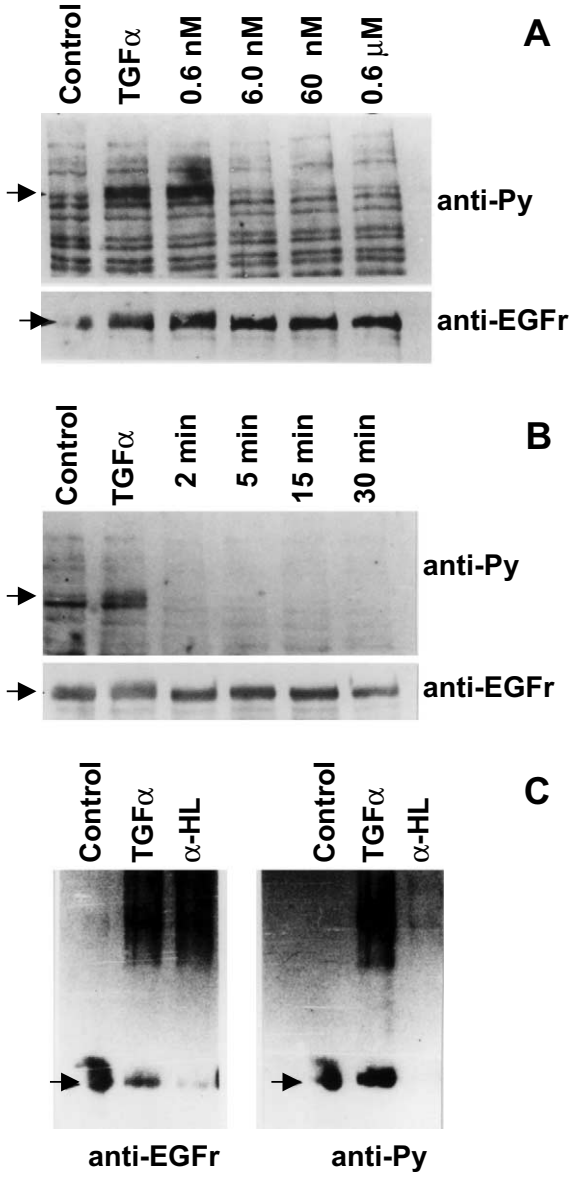

Fig. 2. Diminished phosphorylation of EGFr induced by α-HL at different concentrations and time points. A: Concentration dependence: A431 cells after α-HL treatment were processed as described in Section 2. Throughout the manuscript, the lanes designated as control and TGFα refer to mock treated A431 cells and cells stimulated with TGFα (66 nM) respectively. The phosphorylation pattern was identified by 6G9 anti-phosphotyrosine antibody and the EGFr band was detected by anti-EGFr (sheep polyclonal). The mean density of all EGFr bands present in the bottom panel by taking the TGFα stimulated lane as 100%, is 106 ± 4.8% (except cells labeled control). Throughout the figure the EGFr is marked with an arrow. B: Time course: The A431 cells were incubated with or without α-HL (60 nM)) for indicated time periods followed by stimulation with TGFα and the rest of the experiment is done as described above. The mean density of all EGFr bands present in the bottom panel by taking the TGFα stimulated lane as 100% is 106 ± 13%. C: Absence of phosphorylation of EGFr dimers: A431 cells were treated with α-HL (0.6 μM) followed by stimulation with TGFα. Cross-linking was initiated with glutaraldehyde as described in Section 2. The blot was probed with anti-EGFr antibody (sheep polyclonal) (left) and 6G9 anti-phosphotyrosine antibody (right) as described above.

cannot assemble beyond stage 2. Theoretically, when α-HL binds to target cells, all the three forms viz. the cell bound monomer (stage 2), non-lytic pre-pore (stage 3) and lytic pore (stage 4) can be present on the target cell membrane as only a fraction of the bound α-HL undergoes all the conformational changes to form the lytic pore. In the present study we have examined all these forms (stages 1 to 4) in detail in order to find out which form is responsible for its effect on EGFr of A431 cells. We have exclusively focused our attention on the EGFr phosphorylation because in this event the receptor tyrosine kinase of EGFr, ligand (in the present study it is TGFα), ATP and a phosphatase(s) are believed to be in a dynamic equilibrium. Any change in the status of EGFr phosphorylation can be correlated to the assembly state of α-HL present on the cell surface.

The data in Fig. 1 clearly shows that the α-HL and α-HL(5–293) bind to and oligomerize on A431 cells as efficiently as they do on rRBCs. The H35N, as expected, bound the A431 cells but did not oligomerize. In Fig. 2, the effect of α-HL on the phosphorylation of EGFr is shown. A complete loss of phosphorylation of EGFr was observed with the α-HL concentrations as low as 6.0 nM (Fig. 2A) in 1 h as compared to the cells stimulated with TGFα alone. This effect of α-HL on EGFr phosphorylation was seen within 2–10 min of treatment (Fig. 2B). Also, dimeric forms of EGFr exhibited total loss of phosphorylation signal (Fig. 2C).

The absence of autophosphorylation activity of EGFr in the presence of a pore forming toxin can be due to any one or more of the following possibilities: (i) Depletion of intracellular ATP can occur due to pore formation. Such a depletion of intracellular ATP pool has been shown to down regulate the phosphorylation signal of the EGFr [18]. (ii) Rapid down regulation of the EGFr due to the presence of α-HL, which might result in the inaccessibility of EGFr to TGFα. (iii) Shedding of the receptors due to activation of certain membrane bound proteases. (iv) Perturbation of equilibrium of molecular interactions occurring at the membrane with an effect on EGFr phosphorylation by α-HL. All these possibilities were explored to unravel the mechanism of action of α-HL below.

3.1. Assembly of α-HL is important for the effect

Our earlier studies have shown that the α-HL(5–293) exhibits a significantly retarded cell lysis in case of rabbit red blood cells [11]. We therefore anticipated that it would take a much longer time for α-HL(5–293) to diminish phosphorylation of the EGFr. Interestingly enough, the experiments with α-HL(5–293) showed a total absence of phosphorylation signal of EGFr in the same time range, 2–15 min, as observed for α-HL (Fig. 3A). However, both α-HL and α-HL(5–293) oligomerize on A431 cells as fast as they do on rRBCs. These observations strongly suggest that lytic pore formation and/or membrane damage do not underlie the observed absence of phosphorylation of EGFr. Thus, it is likely that a well defined assembly of the toxin into heptamers may be necessary to cause the loss of phosphorylation of EGFr as both α-HL and α-HL(5–293) exhibited about the same kinetics (Fig. 3B).

There is a 'lag period' in the assembly of α-HL between stages 2 and 4 [1,11–13]. The lysis of cells begins to occur as the pre-pore (stage 3) converts itself to a lytic pore (stage 4). The lag period for α-HL is in the order of several minutes and is further dependent on the toxin concentration and cell type

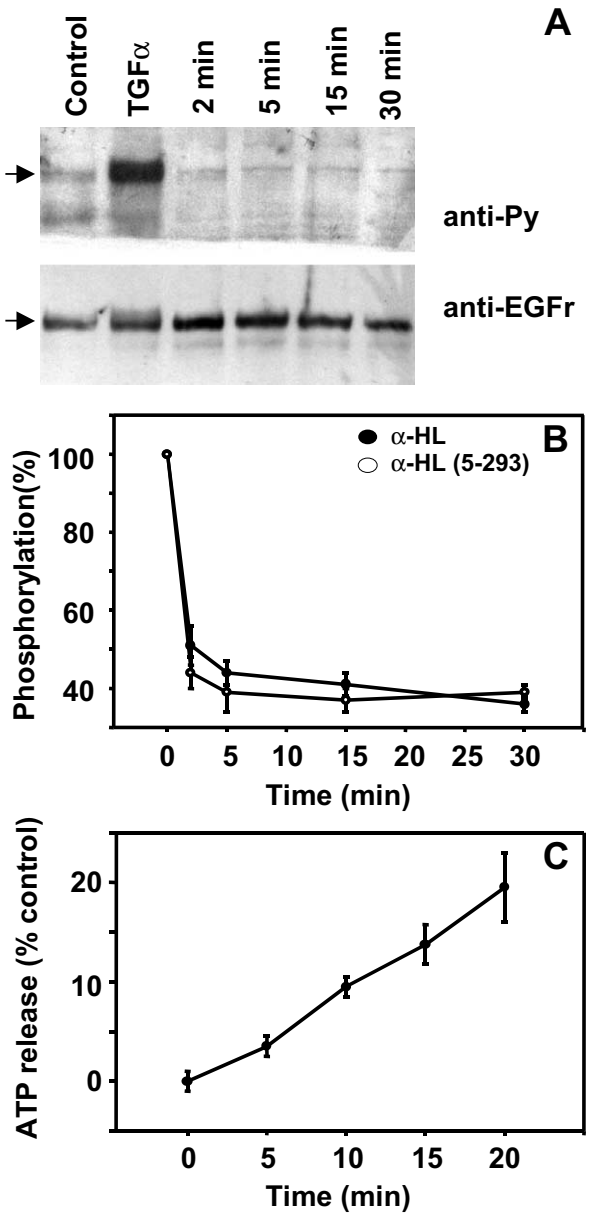

Fig. 3. A: Time dependence of diminished phosphorylation of EGF receptor induced by α-HL(5–293): A431 cells were incubated with or without α-HL(5–293) (60 nM) for different time periods and the rest of the experiment was done as described earlier. The phosphorylation and anti-EGFr patterns were obtained as Fig. 2. The mean density of all EGFr bands present in the bottom panel by considering the TGFα stimulated lane as 100% is 113 ± 9.5%. B: Comparison of kinetics of EGFr dephosphorylation by α-HL and α-HL(5–293): The intensity of phosphorylation signals shown in Figs. 2B and 3A were quantified by densitometric scanning and plotted against time. C: Determination of ATP leakage due to pore formation: The ATP released due to the binding of α-HL was assayed by ATP Bioluminescence kit from Bõehringer-Mannheim. The experiment was carried out as described in Section 2. The data represents the average observations of two different individuals using the same batch of cells at the same time.

(nucleated vs. red blood cells) [11]. The depletion of cellular ATP pool is an unlikely reason for the observed effects in the current experiments for the following reasons: (i) The lag period for α-HL(5–293) is several folds higher than that of wild type α-HL. Thus the membrane damage induced by α-HL(5–293) is insignificant in the time frame of the observed diminished phosphorylation [11]. (ii) Membrane damage is also excluded by our observation that the A431 cells treated with α-HL and α-HL(5–293) at 60 nM concentration do not take up trypan blue for 2 h (less than 1% of cells were stained). This clearly indicates that noticeable membrane damage (due to lytic pore formation) had not occurred during the time frame of diminished phosphorylation. (iii) Identical results were obtained, when exogenous ATP was added to the A431 cells during the α-HL treatment (note that the pore of α-HL is mostly non-selective and bi-directional). (iv) Direct measurement of the amount of ATP leakage due to the presence of α-HL or α-HL(5–293) evoked only marginal loss (about ~10–14%) in the time frame of the diminished phosphorylation observed (Fig. 3C). All these observations strongly support our hypothesis that the observed loss of phosphorylation at the EGFr is not due to the depletion of intracellular ATP pool.

The possibility of down regulation of EGFr occurring within 2–15 min is highly unlikely as the down regulation of EGFr in A431 cells occurs in about 4–6 h, according to Krupp and co-workers [18] and Sturani and co-workers [19]. Shedding of EGFr could be excluded based on our experiments which showed a complete absence of additional low molecular weight fragments of EGFr (data not shown) in the toxin treated cells. This is in sharp contrast to the shedding of IL-6 receptors induced by the treatment of streptolysin O, another pore forming toxin. It is noteworthy to mention here that the shedding of IL-6 was not induced by a streptolysin O mutant that retained cell binding ability but lacked pore forming activity [20]. Our data in conjunction with the streptolysin O data clearly implies that both α-HL and α-HL(5–293) do not affect the integrity of EGFr. In addition, the possibility of membrane bound monomers associating with EGFr through various interactions is also ruled out because treatment of A431 cells with H35N did not result in the diminished phosphorylation of EGFr (Fig. 4A). All these observations point out to the possibility that α-HL indirectly influences the EGFr phosphorylation and the assembly of α-HL is crucial for this effect.

Among several possibilities, the situation presented here is somewhat reminiscent of an earlier observation by Sturani et al. [18]. These authors subjected the EGF stimulated A431 cells to acid wash in order to remove the bound ligand and found that the degree of phosphorylation of the EGFr decreased with time. It is possible in our experiments that it is the phosphorylated EGFr that is affected by α-HL. Hence, it is logical to suspect the involvement of an active PTPase. This is very well in line with the current knowledge on the dynamic control of many signaling cascades by kinases and PTPases associated either with the cell surface or present in the cytoplasm [22,23].

Experiments were carried out in the presence of phosphatase inhibitors in order to examine the role of PTPases in detail. The data presented in Fig. 4B clearly shows that in the presence of sodium orthovanadate the dephosphorylation of EGFr induced by α-HL was greatly reduced or retarded. The reduction in the dephosphorylation induced by α-HL is striking only when the sodium orthovanadate treatment precedes the α-HL treatment, i.e. when cells were first stimulated with TGFα followed by sodium orthovanadate treatment or vice versa. From Fig. 4B (iv), it is also clear that when the cells were treated with the α-HL prior to the treatment with

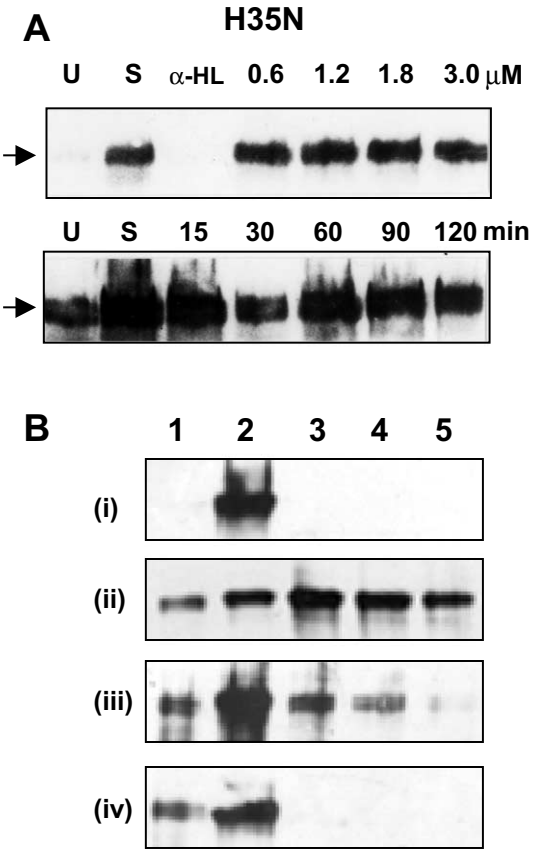

Fig. 4. A: Effect of H35N on EGFr phosphorylation: Concentration dependence (top): The A431 cells were treated with H35N and processed as described above. U, S and α-HL denote unstimulated, TGFα stimulated and α-HL (60 nM) treated A431, cells respectively. The mean density of all the EGFr bands visualized by PY20–HRP by taking the TGFα stimulated lane as 100% is 113 ± 13.5% (the α-HL lane has 7% signal). Time dependence (bottom): The A431 cells were incubated with 1.8 μM H35N for different time periods and the experiment was carried out as described above. The mean density of all the EGFr bands visualized by PY20–HRP (bottom panel) by taking the TGFα stimulated lane as 100% is 92 ± 6.4%. The corresponding anti-EGFr antibody blots showed similar intensities of EGFr with anti-EGFr antibody SC-03 (data not shown). B: Effect of α-HL on EGFr phosphorylation in the presence of the phosphatase inhibitor sodium orthovanadate: Cells were incubated at 37°C for 30 min in the absence (i) and presence (ii) of 1 mM orthovanadate and treated with α-HL followed by stimulation with TGFα for 10 min. The mean density of all the phosphotyrosine bands present in panel B by taking the TGFα stimulated band as 100% is 103 ± 8.9%. (iii) Cells were incubated with 100 mM sodium orthovanadate, stimulated with TGFα followed by treatment with α-HL. Note that lane 5 had less phosphorylation signal for unknown reasons or probably due to cell loss as a result of high concentration of sodium orthovanadate coupled with high concentrations of α-HL and longer incubation times. (iv) Cells were first incubated with α-HL, then treated with 1 mM Na_3VO_4 followed by stimulation with TGFα. In all four panels, lane 1: unstimulated A431 cells; lane 2: TGFα stimulated cells; lanes 3–5: cells treated for 60 min with 50 nM, 100 nM and 200 nM αHL respectively. The phosphorylation pattern was identified by PY20–HRP and the corresponding anti-EGFr blots showed similar intensities of EGFr with anti-EGFr SC-03 (data not shown).

sodium orthovanadate, no reduction in the effect of α-HL was seen. We also examined whether prestimulated receptors can loose their existing phosphorylation signal in the presence of α-HL by using TGFα stimulated A431 cells. Fig. 5A,B shows that there is a dramatic loss of existing phosphorylation of EGFr with time. If prestimulated cells are treated with α-HL followed by treatment with sodium orthovanadate, then the effect of α-HL is only retarded (as seen in Fig. 5A,B; unpublished observations). However, this observation needs to be deciphered further as the effect of α-HL on prestimulated cells is comparatively slow (Fig. 2B vs. Fig. 5B). Interestingly, H35N, a mutant form of α-HL devoid of complete assembly, does not influence the dephosphorylation of EGFr (Fig. 4A). Moreover, this phenomenon of dephosphorylation of EGFr by α-HL occurs in other carcinoma cell lines as well, since the EGFr present in KB (squamous carcinoma, ATCC CCL 17) cell line also did not retain its phosphorylation signal (Fig. 5C). As per the current knowledge, a phosphorylated receptor is dephosphorylated only with the assistance of a PTPase.

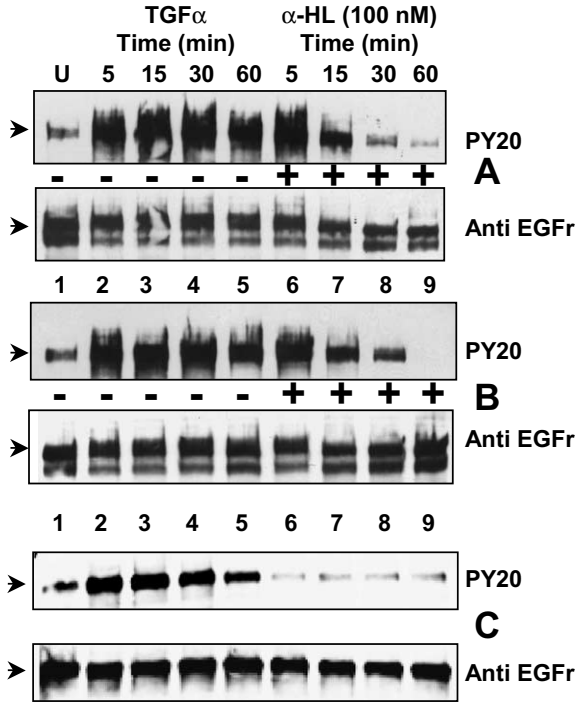

Fig. 5. A,B: Reversal of EGFr tyrosine phosphorylation by α-HL: A431 cells (1.5×10^5) were pretreated with TGFα (70 nM) for 5 min at 37°C. One set was incubated further without addition of toxin ('–' lanes 2–5, panels A and B) and the other set ('+' lanes 6–9, panels A and B) was incubated at 37°C for indicated periods of time. after addition of α-HL (100 nM). For panel A, TGFα was removed before addition of α-HL(100 nM) (lanes 6–9). The mean density of all the EGFr bands present in the bottom panel by taking the TGFα stimulated lane as 100% is 98 ± 9.8%. Lanes 6–9 have 104%, 94%, 49% and 25% phosphorylation signal to that of the respective intensities present in lanes 2–4. For panel B lanes 6–9 α-HL (100 nM) was added without removing TGFα. Lane 1 (A and B) shows control cells (U) incubated at 37°C for 15 min. Lanes 6–9 have 98%, 94%, 70% and 18% phosphorylation signal to that of the respective intensities present in lanes 2–4. The mean density of all the EGFr bands present in the bottom panel by taking the TGFα stimulated lane as 100% is 96 ± 5.4%. The phosphorylation pattern was obtained by PY20–HRP and the EGFr band was detected by SC-03 anti-EGFr antibody. C: Effect of α-HL on phosphorylation of the EGFr on KB cells: The experiment with KB cells is done as with the A431 cells described in Fig. 2. Lane 1: A431 cells alone; lane 2: A431 cells stimulated with TGFα for 10 min; lanes 3–9 the toxin concentrations are 1 nM, 5 nM, 25 nM, 50 nM, 100 nM, 200 nM, 500 nM respectively. The phosphorylation and EGFr patterns were obtained as described above. The mean density of all the EGFr bands present in the bottom panel by taking the TGFα stimulated lane as 100% is 96 ± 5.5%.

Collectively, our data strongly suggests that the effect of α-HL is mediated via a PTPase and is dependent on the oligomerization property of the toxin. One likely mechanism for the observed loss of phosphorylation at EGFr is that immediately after binding, the α-HL engages or activates a PTPase (formation of a molecular ensemble) which may shift the equilibrium of net phosphorylation–dephosphorylation reactions occurring at the EGFr towards dephosphorylation.

Our attempts to identify the PTPase involved were so far unsuccessful, probably due to the extremely low level of expression in vivo. This observation is consistent with the data reported by Pestana and colleagues. These authors could not detect the endogenous rPTPσ in immunoprecipitation and subsequent immunoblotting approaches [24]. In another instance, Tiganis and colleagues had to overexpress the T cell PTPase to detect its cellular target [25,26]. However, in the present context it is absolutely necessary for us to work under the endogenous concentrations rather than with cells lines that overexpress different PTPases. In view of these difficulties it is necessary to design alternate methods to identify, at endogenous concentrations, the PTPase involved in the present context and such attempts are currently underway.

In summary, the data presented here will invoke further interest in understanding the pathogenesis of *S. aureus* in humans in general and in particular the interaction of α-HL and/or similar proteins with certain membrane components present on mammalian cells and their ability to regulate the signal transduction cascades.

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