Signaling through Receptor tyrosine kinases plays a fundamental role in regulation of cell functions like passage through cell cycle, proliferation and differentiation, cytoskeletal organisation and cell-cell interaction. Deregulated kinases can result in proliferative disorders like cancer, psoriasis and atherosclerosis or metabolic disorders like diabetes. On the other hand, certain pathogens have developed sophisticated strategies to interfere with the RTK functions, exploiting these pathways for pathogenesis. It is important to realise that within a cell, tyrosine phosphorylation is a reversible dynamic process. The level of tyrosine phosphorylation in a given protein is regulated by opposing actions of Protein Tyrosine Kinases (PTK) and Protein Tyrosine Phosphatases (PTPs). A balance of both activities plays a crucial role in controlling initiation and termination of receptor based signals

Initial studies conducted in our laboratory have shown that α Hemolysin, a pore forming protein, secreted by *Staphylococcus aureus,* can interfere with the autophosphorylation of Epidermal Growth Factor Receptor (EGFr). Since the pore forming proteins are known to exert their action by punching holes in the cell membrane, it is intriguing as to why such a molecule should interfere with signal transduction and the mechanism by which it brings about the end result. Previous studies from our laboratory using N-terminal deletion mutant (αHL (5-293)) showed that pore formation might not be needed for inhibition of EGFr tyrosine phosphorylation by αHL.

Studies were carried out from this point onward, to study various targets of αHL and to elucidate the possible mechanism of its action on EGFr phosphorylation. We also aimed to answer whether αHL could inhibit the phosphorylation of EGFr in presence of its ligand and whether downstream signaling was affected. A fusion protein between αHL and TGFα (αHLTGFα) was constructed to study how EGFr responds to a molecule which carries both EGFr stimulatory and inhibitory functions in same molecule. Though αHL has been a subject of extensive genetic engineering studies to understand its structure function relationship, no extension mutants of this protein are available. The first aim of the study was to answer whether αHL could be extended at its C-terminal without interfering with its folding and function.

The cloning of αHLTGFα fusion protein, as described in chapter II, required an αHL gene in expressible form, which lacked a stop codon, such that TGFα gene could be placed downstream of αHL. This was achieved by removing the stop codon from an existing αHL clone, and by introduction of an EcoRI site for subsequent cloning. This recombinant vector resulted in αHL (293 amino acids) with a 38 amino extension from the

vector backbone and was designated Carboxy Extension Mutant (CEM). CEM served as an important control in subsequent studies. TGFα gene was PCR amplified with the help of specific primers using TGFα cDNA as template. This gene was fused at 3' end of αHL gene, along with a 21bp linker cassette, to allow maximum flexibility for independent refolding of αHL and TGFα. The sequencing of αHLTGFα and CEM clones showed that sequences were in agreement with those reported for αHL and TGFα. TGFα gene was also fused at the 3' end of gene 10, to yield TC6. Since, in this clone, TGFα was present at C-terminal of an unrelated protein, it served as an important control for EGFr phosphorylation assays in A431epidermoid carcinoma cells.

After cloning of genes, expression of TGFα posed a first major hurdle in the study, as initial attempts to express the gene were unsuccessful, and forced us to reanalyse the gene sequence. Certain codons present in the eukaryotic genes are used at a very low frequency in *E.coli*, resulting in poor expression of the genes. One such rare codon, AGG that codes for Arginine was present in TGFα gene was repaired by PCR mutagenesis, yielding good expression levels of TGFα.

The next step of the study was to optimise the expression of fusion proteins and their purification, as described in chapter III. For preliminary characterisation of the proteins, in *vitro* transcription and translation (IVTT) was used to generate small amounts [^{35}S] Met labeled proteins. Labeling of proteins made their characterisation on various cells simple. For high yield of protein, they were expressed in *E.coli* and purified. IVTT of αHLTGFα invariably resulted in two bands, which matched with those of protein purified from soluble fraction of bacterially expressed protein. Western blot analysis using anti TGFα antibodies against N- or C-terminal of TGFα and peptide mapping by formic acid hydrolysis showed that the cleavage was occurring at the C-terminal of TGFα. A small cleavage at C-terminal of TGFα renders it inactive because it loses the ability to bind EGFr, therefore, it was important to get a full-length αHLTGFα. On expression of αHLTGFα in *E.coli* most of the protein formed inclusion bodies and only about 10% was soluble. The protein in inclusion bodies was not cleaved. Therefore αHLTGFα protein was refolded and purified from the inclusion bodies. αHL portion of the fusion protein does not have any cysteines and is relatively easy to refold and the activity can be checked by simple lysis of rabbit RBCs (rRBCs). TGFα on the other hand carries six cysteine residues involved in formation of three disulfide bonds that are important for TGFα function. Purification protocol therefore involved the refolding in

hampering its folding, rRBC binding, oligomerisation and lytic properties. Such a molecule could form a basis for construction of new molecules in future.

The characterisation of fusion proteins on A431 cells is discussed in chapter5. Both αHLTGFα and CEM form heat and SDS stable oligomers on A431 like αHL. However, the oligomerisation by extension proteins was weak as compared to αHL. Visualisation of oligomers by EM showed well-defined individual pores on A431 membrane, formed by αHLTGFα and CEM. αHL binds more strongly resulting in extensive 2D arrays on A431cells.

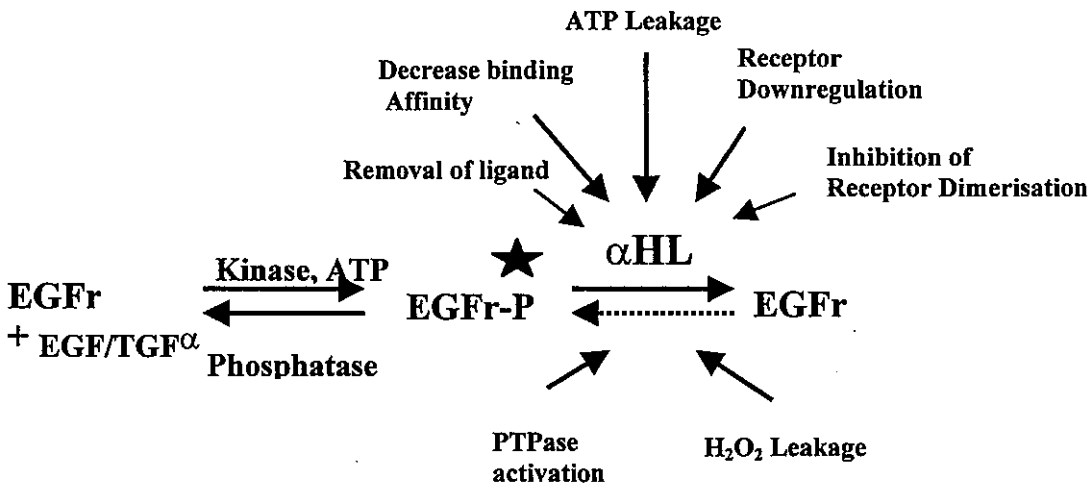
IVTT generated αHLTGFα resulted in two oligomer bands on A431 cells, both of which were heat and SDS stable. While the lower oligomer band was constituted of both cleaved and uncleaved αHLTGFα, the higher oligomer band was constituted predominantly of full-length protein. The fusion proteins were also capable of binding and oligomerising on other cell lines like KB and NIH3T3.

After having proved that both αHL and TGFα were capable of carrying out their respective functions and they could bind and oligomerise on A431and other cells, we aimed to elucidate the mechanism of αHL action on EGFr phosphorylation and to answer how widespread this action was. Intact cells were used for EGFr phosphorylation assays, because all the molecules involved in the event are in their native state. Any change in phosphorylation levels can be correlated with the addition of toxin. The phosphotyrosine content of the receptor can be analysed by western blotting with anti phosphotyrosine antibody.

Incubation of A431 cells with αHLTGFα resulted in initial phosphorylation of EGFr followed by rapid reversal of the signal as is clear from the time course studies on A431 (Section 5.4). With lower concentrations, the reversal required longer incubation times, but was achieved very fast (5-15minutes) at higher concentrations (100nM). This shows that αHL not only was able to inhibit the phosphorylation of EGFr (as concluded from the previous studies) but can even reverse the phosphorylation. The αHL portion of the protein had an important role in dephosphorylation as TC6 fusion protein was unable to bring about dephosphorylation. Prestimulation of EGFr with TGFα, followed by αHL treatment also resulted in rapid loss of Phosphotyrosine signal from EGFr, once again showing that αHL can reverse the stimulation brought about by TGFα. This effect is

regardless of the presence or absence of TGFα. Thus there are two important findings, αHL can reverse the EGFr phosphorylation and it can do so even in the presence of ligand.

Since αHL is a molecule which binds to cell surface and only a part of the glycine rich loop of heptamer protrudes inside the cell, it is fascinating as to how it could affect the phosphorylation of EGFr which is an intracellular event. Inside a cell, the EGFr phosphorylation and dephosphorylation events are in dynamic equilibrium, αHL can exert its inhibitory effect on EGFr phosphorylation through mechanisms outlined below



Plausible Mechanisms of αHL Action

ATP serves as a source for phosphoryl group for kinase reaction and therefore plays an important role in kinase reaction. Depletion of cellular ATP pool due to pores formed by αHL can lead to loss of EGFr phosphorylation. ATP release assays showed that significant ATP depletion had not occurred at the time points and concentration where complete inhibition of EGFr phosphorylation was observed. Moreover, NIH3T3 and KB cells do not leak any ATP even with 1μM concentration of αHL for 1hour and yet show inhibition of PDGFR and EGFr phosphorylation respectively. Finally experiments carried out in detergent extracts showed that αHL could inhibit phosphorylation even in the presence of exogenous ATP, conclusively showing that ATP loss did not have any role to play in rapid dephosphorylation of EGFr.

The possibility that αHL could interfere with receptor dimerisation was ruled by crosslinking studies done previously in the laboratory. To gain insight into whether αHL can interact directly with EGFr and affect its tyrosine kinase activity, experiments were done with detergent extracts prepared by two approaches. In the first case, cells were treated with αHL and then lysed in detergent containing buffer. Stimulation of EGFr by

TGFα was then checked. In the second approach, cell lysates were first prepared and the effect of αHL on EGFr phosphorylation was studied. While αHL inhibited the EGFr phosphorylation in the first case, it was unable to do so in the second case. This may be because the molecular ensemble required for αHL action forms only in intact cells and is not formed in lysates. Alternately αHL may be unable to bind EGFr in the presence of TritonX100.

Since PTPase are involved in reversal of phosphorylation from RTK, αHL can exert its action by synergising with a PTPase. Phosphorylation assays done in presence of PTPase inhibitors, Na_3VO_4 and IAA slowed the effect of αHL and αHLTGFα but could not inhibit it. Experiments done in presence of H_2O_2 showed that αHL was capable of inhibiting non-ligand induced increase in EGFr phosphorylation. The binding of the ligand to receptor results in receptor phosphorylation. The phosphotyrosines serve as docking areas for several downstream signaling molecules. αHL was able to inhibit the phosphorylation of PLCγ, a downstream signaling molecule which associates with EGFr in A431 cells.

In order to understand how diverse αHL action could be, studies were done on other cell lines. KB, an oral squamous carcinoma cell line, which overexpresses EGFr and normal mouse fibroblasts cell line, NIH3T3, which expresses PDGFR were chosen. αHL could inhibit EGFr phosphorylation even on KB cells. Also, the phosphorylation of PDGFR was inhibited.

It is unlikely that a single molecule can have specific interaction with so many diverse molecules present in context of different cellular backgrounds. The results together point towards a unified phenomena i.e. a common system or presence of an accessory molecule on cell surface which is aiding in αHL function. The accessory molecules, which can aid αHL function, can fall into 3 classes

1. PTPase
2. Protein Kinase C
3. gangliosides

*Since an existing phosphorylation signal can be removed only by a PTPase, it is surprising that a PTPase inhibitor failed to block αHL action. Relatively little is known about PTPases, which fall into two categories, transmembrane and cytosolic. αHL by interacting with a Receptor like transmembrane PTPase can cause conformational

changes altering their activity. Whether αHL can recruit cytoplasmic PTPase is difficult to speculate. Such a PTPase can in turn act on the diverse targets of αHL.

*Many of the membrane perturbing agents like δ Hemolysin (δ-HL) and mellitin result in activation of protein Kinase C. αHL by activating a PKC can bring about the end result by interfering with the forward (kinase)as well as backward (PTPase) reaction, since PKC can modulate both the activities by Ser/Thr phosphorylation. EGFr is known to undergo a ser/thr phosphorylation, resulting in a decrease in its tyrosine kinase activity. PKC is also involved in Ser /Thr phosphorylation of certain PTPases which enhance their *in vitro* activity.

*Thirdly, Glc NAc ganglioside has been proposed to be the receptor for αHL on cell membranes. Gangliosides in turn are potent regulators of EGFr and PDGFR function and ganglioside, GM3 has been shown to stimulate RPTPσ by an unknown mechanism. αHL by interacting with these molecules can lead to inhibition of EGFr phosphorylation. Alternately, the membrane perturbing action due to binding and changes in lipid microenvironment may be modulating the EGFr function.

The results together show that αHL is a potent inhibitor of receptor tyrosine kinase function. It can not only inhibit RTK stimulation by its ligand but bring about reversal of already phosphorylated receptor. The action of αHL is diverse in that it can inhibit multiple RTK. αHL also shuts off the downstream signaling events.

Most of the natural RTK inhibitors eg Erbstatin, Quercitin and synthetic ones eg PD135035, tyrphostins are ATP or protein substrate or bisubstrate analogs. Cell permeabiliy is an important criteria for successful inhibition by these inhibitors. Competetive inhibitors could be less effective in inhibiting the autophosphorylation step because in autophosphorylation reaction the substrate concentration is high due to proximity effect. Therefore, though these inhibitors may inhibit phosphorylation of exogenous substrates, they may be poor in inhibiting the phosphorylation of EGFr. αHL meets most of the requirements for generation of a potent RTK inhibitor. One, it intercepts at the very first step in signal transduction, i.e autophosphorylation of the receptor. Secondly, it can do so in intact cells. Third, it acts from outside the cells and therefore does not pose any problem of permeability. Four, It not only inhibits the autophosphorylation of the receptor but switches off the downstream signaling events. Finally, The presence of ligand cannot reverse the action of αHL.

Studies on mechanism of αHL action can help gain further insight into pathogenic strategies employed by *Staphylococcus aureus*. αHL is secreted as a water soluble molecule, which forms transmembrane pores. However it has been conclusively shown to inhibit EGFr phosphorylation. The fact that αHL can inhibit the EGFr phosphorylation may be of relevance to the fact that *Staphylococcus aureus* causes dermonecrosis, as EGFr is predominantly expressed on skin cells. αHL could be acting on the cells by forming pores or by interfering with signal trasduction or both.

Pathogens/ parasites have become masters at manipulating the structure and pathways of host cell for their own noxious/baneful purposes. Evolution has played a great role in generation of novel molecules which can act in much more spectacular manner e.g. PTPase secreted by Yersinia is by far the most active PTPase known with the Kcat value 25 fold higher than that of mammalian PTP-1 enzyme. The 'smart' molecules produced by pathogens can be used as templates/models to engineer molecules for therapeutic purposes. While αHLTGFα fusion protein served as a tool in understanding the effect of αHL on EGFr function, molecules can be designed based on αHL that can interfere with the signal transduction pathway in a much more dramatic way. It may be possible to specifically target molecules like αHL to their target receptor, where they can shut off cell signaling pathway leading to growth inhibition.

αHL can serve as a potent tool to study *in vitro* signal transduction events in the near future. Insights into mechanism of αHL action and cell biology studies along with the genetic tools may help in generation of molecules, which can serve as potent PTK inhibitors, which can act from outside the cells.