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The Influence of a Coiled-coil Isoleucine Zipper on the Trimerization and Endosomolytic Activity of the E5-TAT-mCherry Protein

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The Influence of a Coiled-coil Isoleucine Zipper on the Trimerization and Endosomolytic Activity of the E5-TAT-mCherry Protein

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ABSTRACT

The E5-TAT-mCherry protein is designed to initiate cellular uptake through endocytosis and react to the acidification of the endosomes by causing membrane lysis and cytosolic delivery. The cell penetrating peptide TAT from the HIV transcription activator binds to the cellular membrane and transports attached or coincubated cargo into the cell through endocytosis. E5 is a derivative of the fusogenic peptide HA2 from the influenza hemagglutinin protein and reacts to the acidification of the endosomal lumen by inserting into the membrane and increasing membrane permeability. The attached red fluorescent protein mCherry serves as a convenient biological probe for cell based assays. Cytosolic delivery has been achieved using E5-TAT-mCherry, although efficiency still remains low. It is thought that imitating the triple stranded coiled coil state of the hemagglutinin protein will lead to increased membrane lysis and greater cytosolic delivery. Therefore, in order to create a more native-like state and to increase the local concentration of the protein within the endosome, a pH-sensitive isoleucine zipper (IZdep) has been incorporated into the sequence. Under acidic conditions, such as those within endosomes, the pH-dependent IZ forms a triple stranded coiled coil. E5-TAT-IZdep-mCherry was expressed in *E. coli* and purified with chitin beads and ion exchange chromatography. Native PAGE analysis indicates that two oligomerization states exist for E5-TAT-IZdep-mCherry, however, gel filtration shows only one band. An *in vitro* erythrocyte lysis assay was used to determine the amount of membrane lysis induced by the protein complex. Very little hemolysis was observed, and surprisingly, microscopic images indicated that the protein did not bind to the inner leaflets of the RBC ghosts as was expected. These results indicate that IZ is not effectively causing the trimerization of the mosaic protein and that the positively charged TAT peptide is interacting with the negatively charged residues on the IZ sequence which decreases the membrane binding efficiency.

INTRODUCTION

Cell penetrating peptides present an attractive approach forthe cytosolic delivery of macromolecules. Because membrane translocation is limited to highly hydrophobic and small uncharged molecules, alternative methods of delivery are needed to deliver large or charged molecules across the cellular membrane. Cell penetrating peptides (CPPs)

are a group of molecules that may be used to deliver cargo into live cells. TAT(GRKKRRQRRR) is a common CPP that is derived from the HIV Trans-Activator of Transcription protein. TAT is positively charged and will bind to a negatively charged membrane. By binding to the cellular membrane TAT can trigger endocytosis. Attaching a large or hydrophilic cargo to TAT will result in the endocytosis of the cargo into the cell (1).

However, once inside the endosome there is low efficiency of delivering the cargo to the cytosolic space. The TAT peptide and cargo then become trapped inside of the endosome (2). A peptide with membrane lytic ability can be used to disrupt the endosomal membrane creating space for the cargo to escape (3). One such peptide is HA2 from the influenza hemagglutinin protein. Hemagglutinin naturally forms a triple stranded coiled coil with the HA2 fusogenic peptide pulled in toward the center of the protein. Under acidic conditions hemagglutinin changes conformation and HA2 becomes activated (4). The protein then exposes the activated HA2 peptide to the external environment where it can insert into a membrane and cause lysis (5). Derivatives of HA2 have been linked to the N-terminus of TAT in order to combine the cell penetrating ability of TAT with the membrane lytic activity of HA2. E5 is an HA2 derivative that has shown superior membrane lytic ability to HA2 itself (6).

Together HA2 derivatives and TAT form a powerful chimeric peptide. The conjugation of a fluorescent protein to the C-terminus of TAT allows the cytosolic and endosomal distribution of the peptide to be visualized (7). The fluorescent protein must be stable under neutral and acidic pH in order to withstand the acidification of the endosomal space. It also must be biologically inactive. mCherry was chosen as a suitable

fluorescent protein. In addition to meeting the listed requirements, mCherry exists as a monomer and therefore will not cause a mixture of oligomerization states (8, 9).

The complete protein E5-TAT-mCherry has been used to deliver dextrans to cells. The dextrans, when coincubated with E5-TAT-mCherry, were successfully endocytosed and then released from the endosomes. However, the endosomal release was still inefficient compared to E5-TAT and it is suspected that the endosomolytic activity of E5-TAT-mCherry was not sufficient to achieve cytosolic release (7).

Membrane lysis by E5 is dependent upon a high local concentration of the peptide congregating at the endosomal membrane.An increase in local concentration of fusogenic peptide may be achieved by attempting to mimic the natural state of the hemagglutinin protein. It has been shown that attaching a coiled coil peptide to a fusogenic peptide increases the membrane lytic activity of the peptide (10).The assembly of a coiled coil is based upon the formation of a hydrophobic core as well as electrostatic interactions between oppositely charged residues on adjacent peptide chains (11). The isoleucine zipper (IZ) consists of a heptad repeat (IEKKIEA) that self assembles into a triple-stranded coiled coil with a native-like structure (12). A pH-dependent isoleucine zipper (IEKKEEA) also exists. This peptide presents the possibility of causing the pH dependent trimerization of the fusogenic peptide, E5. Under neutral conditions the pH-responsive IZ remains monomeric; however, when placed in an acidic environment the peptide forms a triple stranded coiled coil.

MATERIALS AND METHODS

Expression and Purification of E5-TAT-IZind-mCherry and E5-TAT-IZdep-mCherry

The plasmid pTXB1-SUMO-E5-TAT-IZ (ind and dep)-mCherry-CBDwas obtained from previous studies. The protein contained a small ubiquitin-like modifier (SUMO) protein at the N-terminus to prevent the lytic E5 peptide from causing any damage during expression. Also, a chitin binding domain (CBD) was attached to the C-terminus to allow for purification with a chitin bead column. The plasmids were transformed into BL21 (DE3) *Escherichia coli* cells and protein expression was induced with the addition of 1X IPTG (1 mM) at 16°C for 48h. Cells were centrifuged at 4,000 rpm for 30 min and then resuspended in pH 7.5 lysis buffer containing 20 mM tris(hydroxymethyl)aminomethane and 200 mM NaCl supplemented with SIGMAFAST protease inhibitor cocktail tablets. Cells were sonicated for 5 min cycles with alternating 5 sec pulse on and 5 sec pulse off. A set of 3 cycles was performed. Lysed cells were centrifuged at 13,000 rpm for 45 min. The supernatant containing SUMO-E5-TAT-IZ-mCherry-CBD was passed through a 0.22 µm filterand added to a chitin bead column pre-equilibrated with lysis buffer.The column was gently mixed to suspend the beads in the buffer then incubated at 4°C overnight. The beads were washed with 3 column volumes (Cv) of lysis buffer. The protein was cleaved from the chitin beads by adding 1 Cvof lysis buffer supplemented with 100 mM sodium 3-mercapto-1-propanesulfonate and incubating overnight at 4°C. The eluted SUMO-E5-TAT-IZ-mCherry protein was concentrated using a membrane with a 10 kDa cut-off. The lysis buffer was exchanged with ion exchange (IEx) buffer containing 50 mM HEPES at pH 7. 2 µL of SUMO protease (1U/µL) was incubated with the protein for 2 hrs at room temperature to cleave the SUMO tag.The proteins were

passed through a 0.22 μm filter, and the filtered samples were further purified by ion exchange chromatography using a HiTrap SP HP column and IEx buffer A containing 50 mMHEPES at pH 7 and IEx buffer B containing 50 mM HEPES and 2 M NaCl at pH 7. The eluted protein fractions were analyzed by SDS-PAGE. Finally the fractions containing E5-TAT-IZind-mCherry and E5-TAT-IZdep-mCherry were recovered as final products.

Hemolysis Activity Assay

A membrane lysis assay was performed with human erythrocytes. The red blood cells (RBCs) were washed with 1X DPBS and centrifuged at 1500*g* for 5 min to remove the lysed RBCs. The wash procedure was repeated 6 times. RBCs were diluted in buffer, either 1X DPBS at pH 7.2 or citric acid saline at pH 5, to 1.25%. Protein was added to a final concentration of 1 μM. The RBCs were incubated with protein for 30 min at 37°C. The sample was then centrifuged at 1500*g* for 5 min and the supernatant was transferred to a 96 well plate. The absorbance of hemoglobin was measured at 450 nm in order to determine the level of hemolysis for each protein. RBCs mixed with 1% TritonX-100 were used as a positive control. RBCs mixed with IEx buffer A alone served as a negative control.

NativePAGE

Native PAGE was performed using the standard procedure for a 12% SDS-PAGE, substituting water for SDS. The running buffer was prepared containing 192mM glycine and 25 mM Tris base. Fluorescent images were obtained using a Typhoon TRIO Imager. The gel was excited with a green laser (532 nm) and used a 610 BP emission filter for detection.

Gel Filtration

Gel filtration was performed by using a Superdex 75 PC 3.2/30 column. A 25 µL sample volume and a 0.05 mL/min flow rate were used for each run. Buffer containing 25 mM HEPES, 100 mM NaCl, and 100 mM 1,6-hexanediol was used for pH 7 and buffer containing 25 mM Citric acid, 100 mM NaCl, and 100 mM 1,6-hexanediol was used for pH 5.

Microscopy Assay

RBCs were incubated with different concentrations of protein on the inverted epifluorescence microscope (Model IX81, Olympus, Center Valley, PA) equipped with a heating stage maintained at 37°C. Cells were allowed to settle to the bottom of the wells for 5 min to obtain a layer of cells in the focal plane. Images were collected using bright field imaging and one standard fluorescence filter, theIRFP filter (Ex:535-580, Em:590-670).

RESULTS

Protein Purification

A pH independent and a pH dependent form of the isoleucine zipper were each added into the protein sequence and expressed separately. Fractions of expressed protein containing the pH independent isoleucine zipper, SUMO-E5-TAT-IZind-mCherry-CBD and purified SUMO-E5-TAT-IZind-mCherry were collected and analyzed by SDS-PAGE (Fig. 1). A band corresponding to SUMO-E5-TAT-IZind-mCherry-CBD is seen on the gel. From purified samples a band appears at about 50 kDa which corresponds closely to the expected molecular mass of 47.4 kDa for SUMO-E5-TAT-IZind-mCherry.The same procedure was followed for the protein containing the pH sensitive isoleucine

zipper, SUMO-E5-TAT-IZdep-mCherry, and a corresponding band was also visualized on a gel (data not shown).

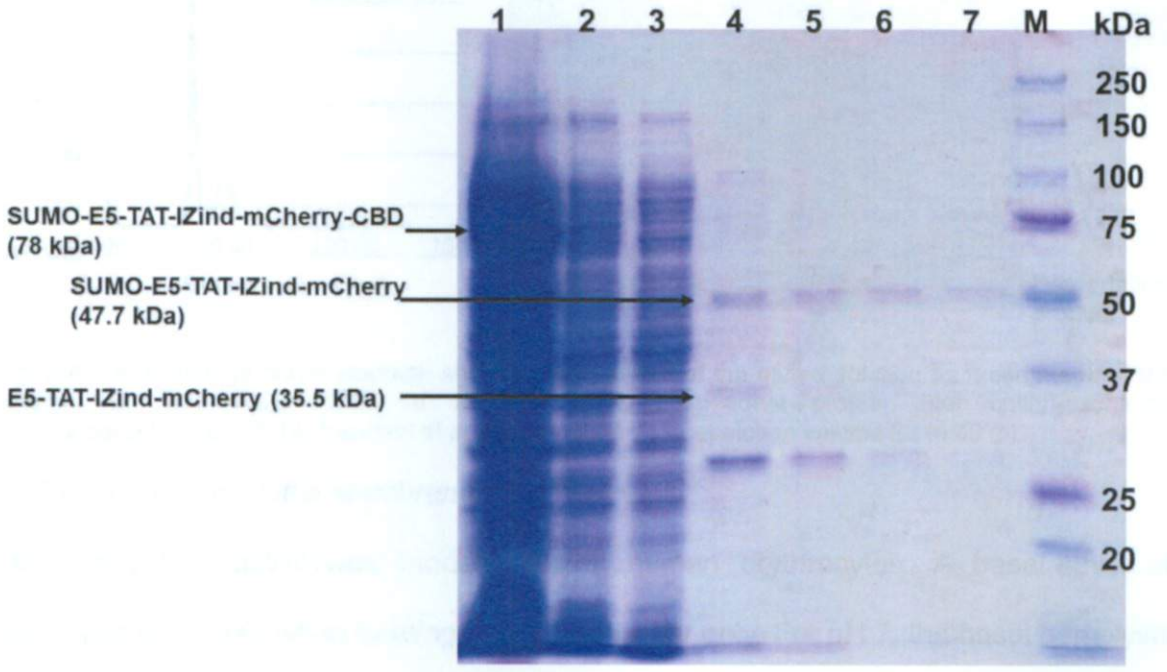


Fig. 1.SDS-PAGE (12%) of IZ-independent protein purification. Lane 1: Crude proteins just after harvesting cells. Lane 2: Chitin beads flowthrough. Lane 3: Chitin beads wash, Lane 4-7: Fractions of cleaved protein from chitin beads

After the SUMO tag was cleaved both the protein containing the pH dependent and pH independent isoleucine zipper were purified by ion exchange chromatography, fractions were analyzed by SDS-PAGE (Fig. 2). A band was seen with a similar molecular mass to the expected mass of E5-TAT-IZind-mCherry, 35.5 kDa. There also appeared to be a degradation product with a mass below 20 kDa. It is important to note that this degradation product was only seen when samples were boiled. Several unboiled samples were run on SDS-PAGE and little degradation was seen (data not shown).

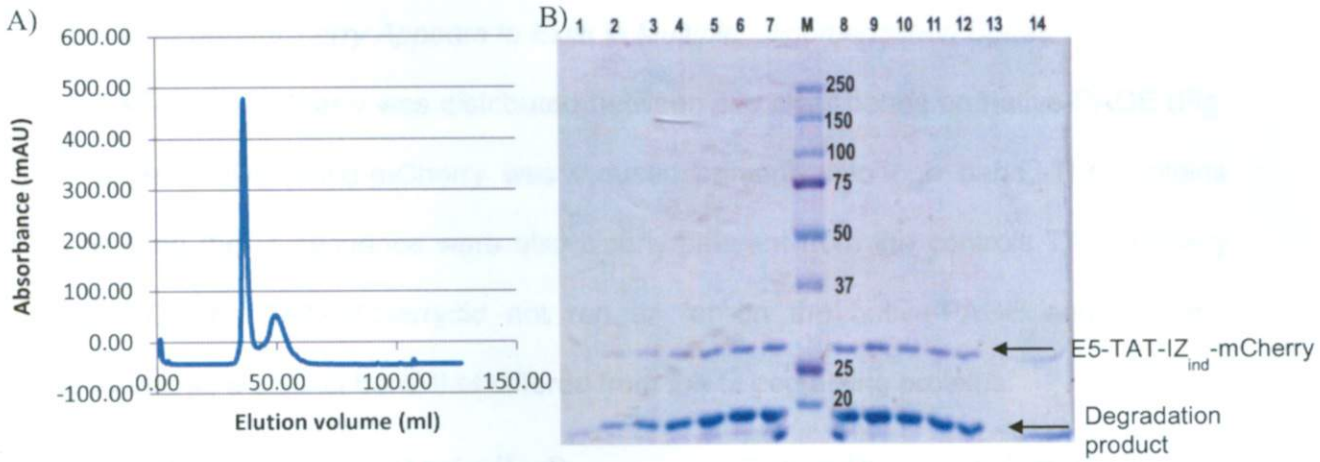


Fig. 2.A. Ion exchange chromatograph showing absorbance of the eluted solution as it comes off of the column. B. SDS-PAGE (12%) of fractions of IZ-independent protein after cation-exchange chromatography. Lane 1-14: fractions of protein from the peak at elution volume 32 to 40 ml.

IZ Proteins Display Little Membrane Lytic Activity

Membrane lytic activitywas modeled using human erythrocytes. A basal level of hemolysis was seen when treating RBCs with buffer only. For pH 7, the basal hemolysis was 9% while at pH 5 basal lysis increased to 31%. Unfortunately the lysis shown by the IZind and IZdep was below the basal level under both pH conditions. This assay was repeated twice with similar results each time.

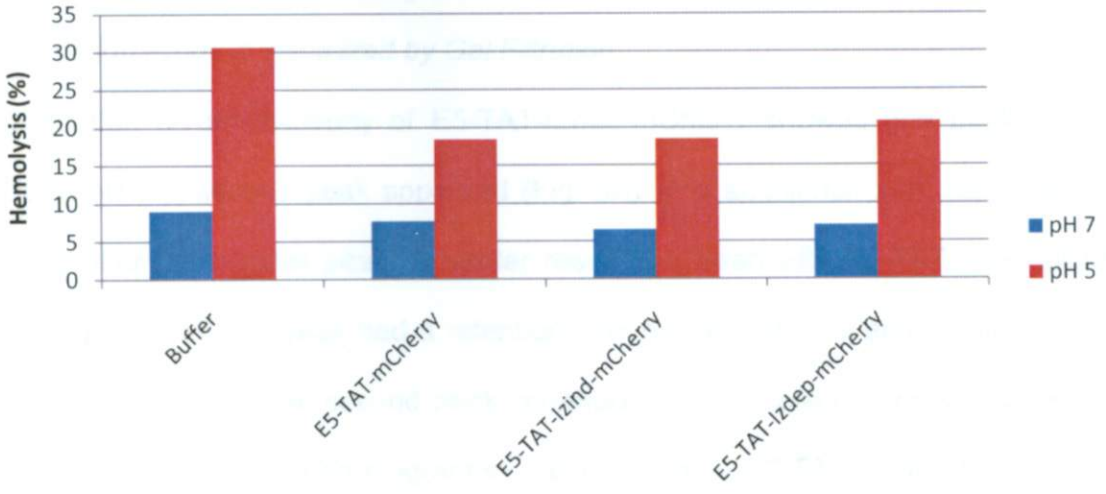


Fig. 3.IZ proteins induce no significant lysis. Hemolytic activities of E5-TAT-mCherry, E5-TAT-IZind-mCherry, and E5-TAT-IZdep-mCherry at pH 7.0 and 5.0 for 1.25% RBC suspensions

E5-TAT-IZdep-mCherry Appears to Exist in Multiple Oligomerization States

E5-TAT-IZdep-mCherry was distributed between two clear bands on native-PAGE (Fig. 4) while E5-TAT-IZind-mCherry was focused primarily into one band. The proteins containing the IZ sequence were also clearly different from the controls TAT-mCherry and mCherry. TAT-mCherrydid not run as far on the native-PAGE and mCherry appeared as a distinct band that differed from the IZ containing proteins.

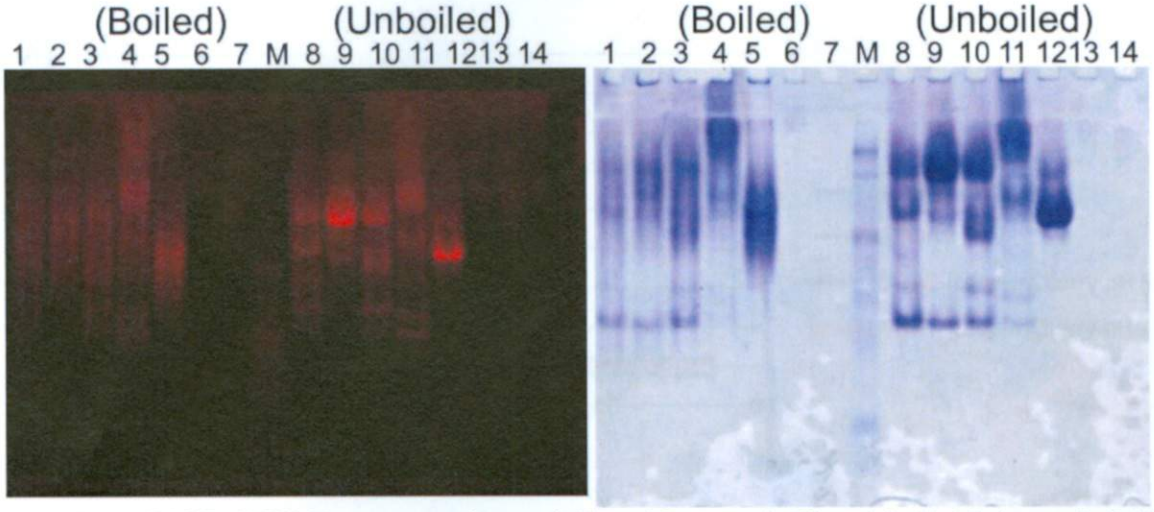


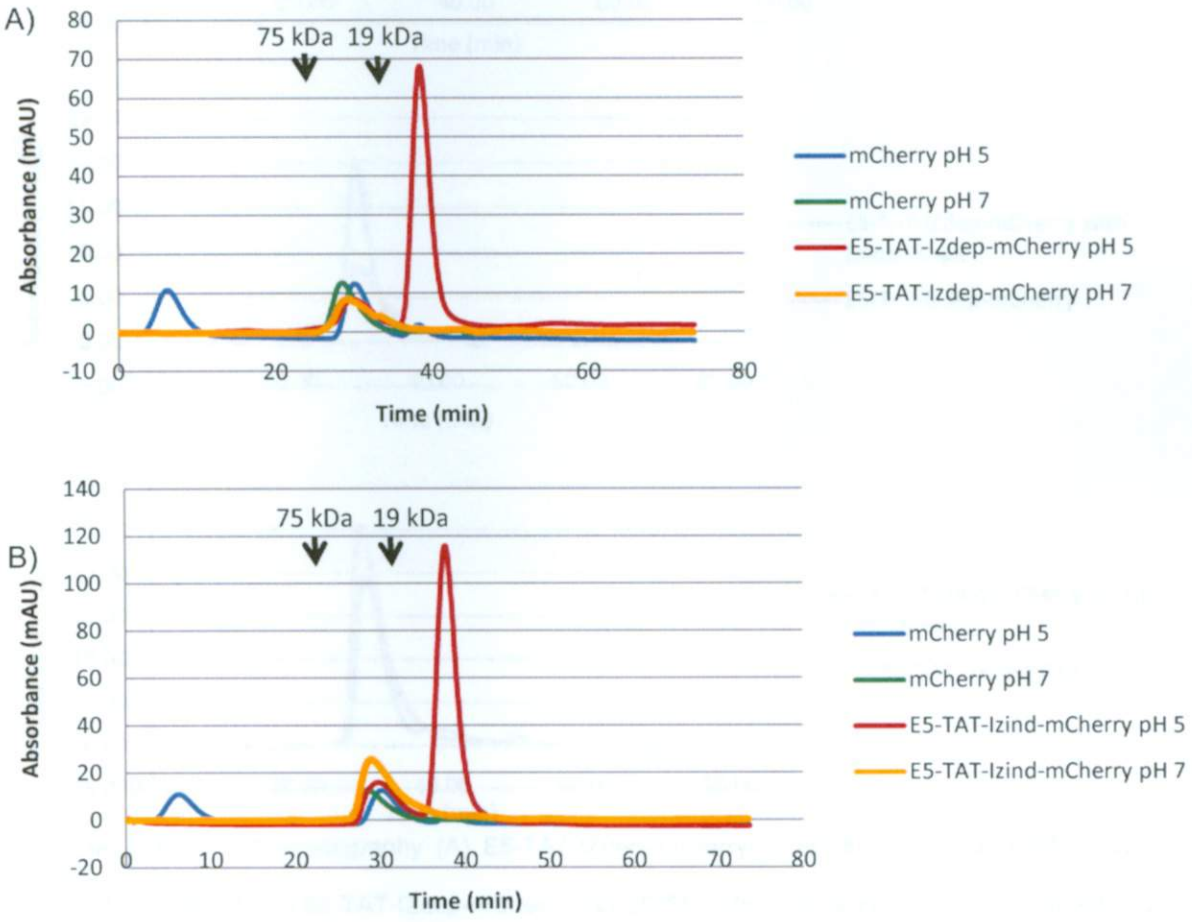
Fig. 4. NativePAGE. (12%) Fluorescence image (left) and Coomassie Blue stain (right). Lanes 1, 8: E5-TAT-mCherry; 2, 9: E5-TAT-IZind-mCherry; 3, 10: E5-TAT-Izdep-mCherry; 4, 11: TAT-mCherry; 5, 12: mCherry; 6, 7, 13, 14: Gel Filtration Degradation Peak

Degradation Product Visualized by Gel Filtration

Gel filtration chromatography of E5-TAT-IZdep-mCherry showed one peak at pH 7, while at pH 5 a second peak appeared (Fig. 5A). The additional peak came off of the column after the original peak. A similar result was seen with E5-TAT-IZind-mCherry (Fig. 5B). The original peak had a retention time between the 75 and 19 kDa markers. The retention time of the second peak corresponds to a molecular mass that is below 19 kDa. The chromatographs comparing E5-TAT-IZind-mCherry and

E5-TAT-IZdep-mCherrywere very similar to each other, with differences only in peak intensity (Fig. 5C). This was true at both at a neutral pH and an acidic pH.

Gel filtration was run with a heparin salt solution (Fig. 5D). There was no significant difference between the peaks from the solution with heparin and without heparin. Gel filtration was also run using a buffer without 1,6-hexanediol (Fig. 5E). There was also no significant difference between the retention times of the protein in a solution with or without 1,6-hexanediol.



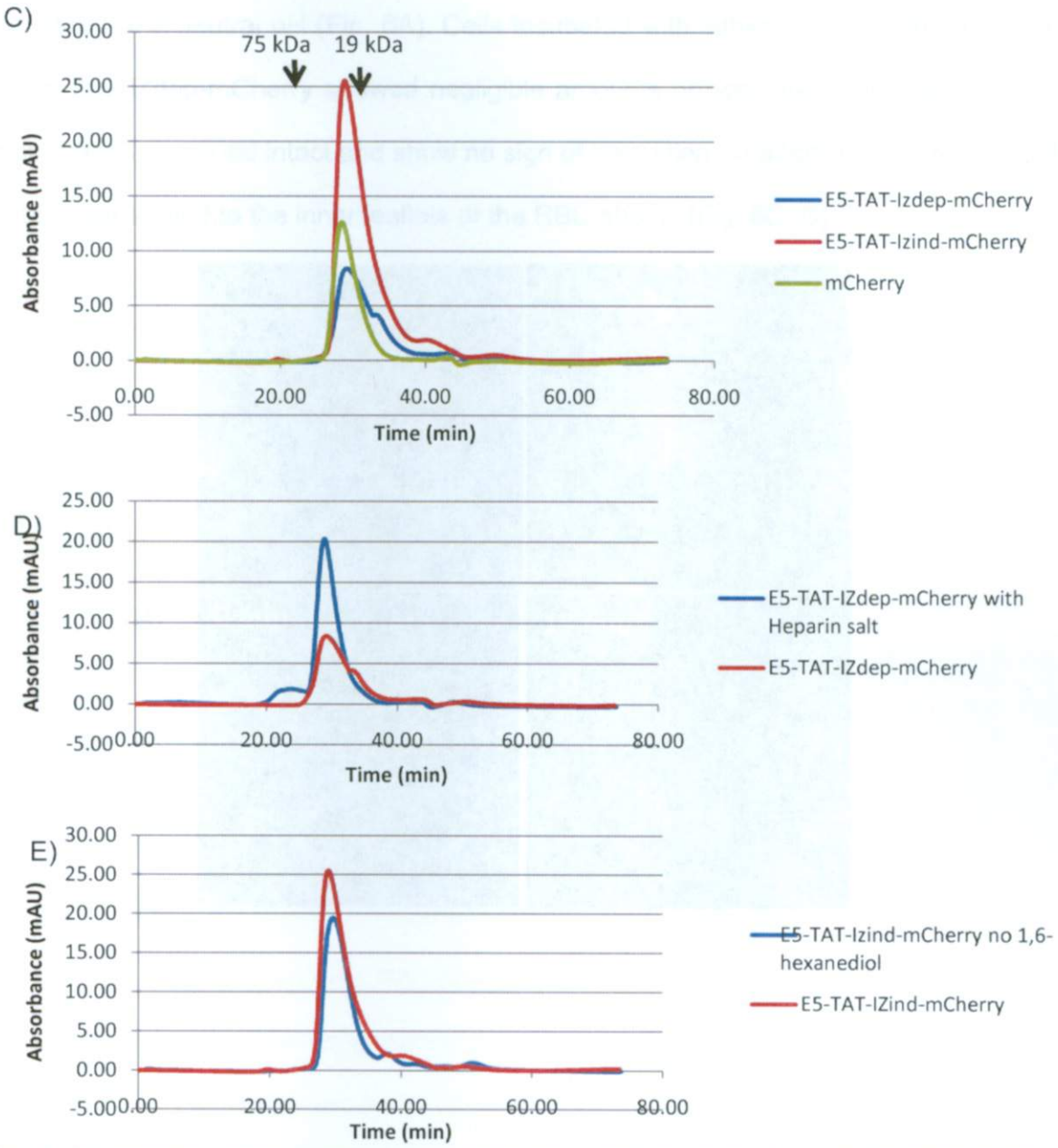


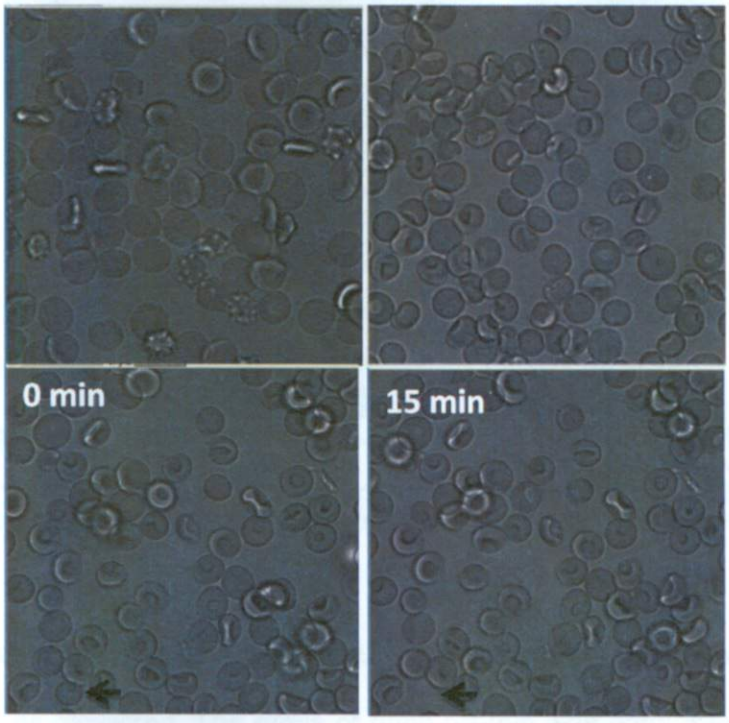
Fig. 5. Gel Filtration chromatography (A) E5-TAT-IZdep-mCherry at pH 5 and 7. (B) E5-TAT-IZind-mCherry at pH 5 and 7. (C) E5-TAT-IZdep-mCherry and E5-TAT-IZind-mCherry at pH 7. (D) The effect of heparin salt on E5-TAT-IZdep-mCherry. (E) The effect of 1,6-hexanediol on E5-TAT-IZind-mCherry

Microscopy Assay Reveals Little Hemolytic Activity

Red blood cells incubated at low pH appeared to be more spherical than RBCs incubated at a neutral pH (Fig. 6A). Cells incubated with either E5-TAT-lZind-mCherry or E5-TAT-lZdep-mCherry showed negligible amounts of hemolysis (Fig. 6B). Cellular membranes remained intact and show no sign of disruption. In addition, the proteins did not appear to bind to the inner leaflets of the RBC ghosts (Fig. 6C, D).

A)

B)



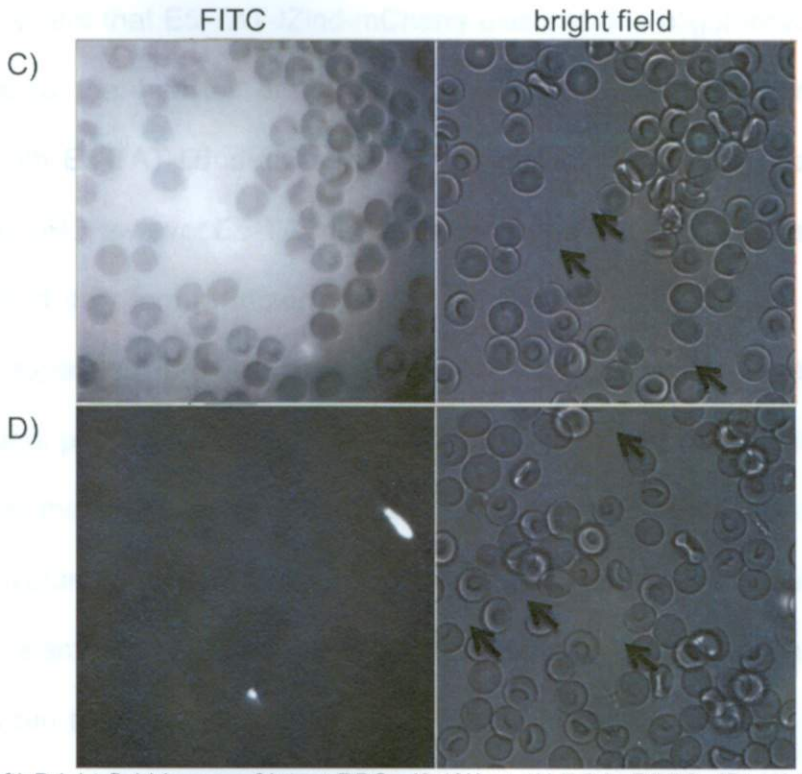


Fig. 6. (A). (left) Bright field image of intact RBCs (0.1%) at pH 7.2 in DPBS, (Right) Bright field image of RBCs (0.1%) at pH 5 in citric acid buffer. (B) Bright field image of RBCs and E5-TAT-IZdep-mCherry (1 uM) with RBCs (0.1%) at pH 5 (left) at 0 min and (right) after 15 min (C) E5-TAT-IZind-mCherry (1 uM) with RBCs (0.1%) at pH 5. (D) E5-TAT-IZdep-mCherry (1 uM) with RBCs (0.1%) at pH 5

DISCUSSION

Our data indicates that we have isolated our proteins of interest; however the proteins are not behaving as expected. The mass of each protein was confirmed by SDS-PAGE. The activity of the protein was then determined in order to see if the addition of the IZ coiled coil sequence would increase the membrane lytic ability of the protein. The results of the hemolysis assay, however, indicated that both IZ containing proteins caused an insignificant amount of hemolysis. Both IZ proteins were then run on a native-PAGE in order to determine how the IZ sequence could be affecting the hemolytic ability and oligomerization state of E5-TAT-mCherry. The native-PAGE seemed to confirm the existence of two oligomerization states for the IZ-dependent

protein. It appears that E5-TAT-IZind-mCherry exists in one oligomerization state which corresponds to the heavier of the two states for E5-TAT-IZdep-mCherry. This is consistent with E5-TAT-IZind-mCherry forming one higher order oligomerization state regardless of pH. However E5-TAT-IZdep-mCherry is designed to remain a monomer at neutral pH and only form a trimer under acidic conditions. The native PAGE suggests that the IZ-dependent protein forms some kind of higher order oligomerization state even at neutral pH. However,with a nativePAGE there are several variables which may affect how far the bands travel on the gel and therefore the proteins were run through a gel filtration column in order to obtain more definitive results. The benefit of gel filtration is that the retention time depends only upon the size of the protein. When each IZ protein was run through gel filtration at pH 7 the main peak appeared to correspond to mass of the monomeric protein. There was no significant difference in retention time between the IZ-independent and the IZ-dependent protein. The additional peak that appeared under acidic conditions appearsonly to be a degradation product with a mass less than 19 kDa. It is suspected that TAT, because it is positively charged, may interact with the negatively charged residues on the IZ sequence and prevent the protein from self-assembling into a coiled coil. In order to investigate this idea heparin salt was added to the gel filtration buffer. Heparin salt is a known TAT inhibitor and binds to the positively charged residues. If the addition of heparin salt aids in the formation of a coiled coil it could be concluded that TAT indeed was interfering with the coiled coil formation. However, the addition of the salt did not cause any change in retention time or any additional peaks to elute from the column. Another method was employed to encourage coiled coil formation-the 1,6-hexanediol was removed from the gel filtration

buffer. In order for the IZ sequence to form a coiled coil the hydrophobic residues of the IZ sequence must be oriented toward the center of the coil. It was hypothesized that 1,6-hexanediol, which contains a hydrophobic chain, might be preventing the hydrophobic IZ core from forming. Yet even with the removal of 1,6-hexanediol from the gel filtration buffer the elution pattern remained the same.

A closer look was then taken at the hemolysis assay. RBCs were monitored under a microscope in order to determine if any interactions were occurring between the proteins and RBCs. Surprisingly the IZ proteins did not bind to the RBC ghosts. The positively charged TAT sequence was expected to bind to the inner leaflets of the lysed RBCs, yet no interactions were apparent. It can be concluded that there is interference present within the protein structure itself preventing the formation of a trimeric coiled coil. This interference is also contributing to a decreased level of hemolytic activity and membrane lysis in comparison to earlier studies with E5-TAT-mCherry. The interference is likely due to the positively charged TAT peptide interacting with negative charges elsewhere on the protein, especially the negative residues in the IZ sequence. It would be beneficial to remove the TAT sequence from the protein in order to confirm this interaction. If an E5-IZ-mCherry protein forms a trimer it can be concluded that TAT is responsible for preventing the oligomerization of the proteins. It is also suspected that increasing the number of heptad repeats within the IZ peptide would increase the ability of the peptide to form a coiled coil. In this situation, even if TAT interfered with part of the IZ peptide, the longer sequence would still allow for coiled coil formation. Further studies are required to address these concerns.

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