The Effects of Linker Length and Flexibility on Fc-Fusion Proteins

The Effects of Linker Length and Flexibility on Fc-Fusion Proteins

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Abstract

Aggregation, yield, and overall stability of molecules are of enormous importance for development of biopharmaceuticals. Several construct and cell-based optimization approaches are being undertaken to generate molecules with increased stability and yield. Different element of the DNA construct can affect expression and aggregation levels of a protein, including the type of linker used to connect the extracellular domain to the Fc of a fusion molecule. The choice of linker is protein specific depending on the function of the molecule and may require distance between the domains for correct folding. The aim is to examine the effects of linker rigidity and length on folding, yield, and aggregation of ActRIIB-Fc homodimer. In particular, we selected hIgA1 hinge and (AP)x11 from literature as the two linkers to test, due to differences in structure, rigidity, and composition. In this study, ActRIIB-Fc was cloned with hIgA1 hinge and (AP)x11, two linkers with rigidity added to its structure due to proline residues, along with two flexible control linkers composed of GGG and (G4S)4. All four constructs were transiently expressed in ExpiCHO cells and purified using protein A affinity resin followed by Preparative SEC. The quality of each product was analyzed for changes in titer, aggregation and other contaminants in culture by western blots and size exclusion chromatography (SEC). They were further characterized for binding and inhibition of its interacting partners. Proteins were also assessed for any changes in thermal stability and folding due to the rigidity and length of the linkers. We observed an improvement in overall affinity of the product to a proprietary anti-ActRIIB antibody affinity column.

Evaluation of the cultures revealed no change in aggregation between the rigid and flexible linkers but there was an increase in low molecular weight species, which translated to a lower percentage of correct size molecule, seen with rigid linkers. There was no change in binding and inhibition of the ligands, and we noted no difference in thermal stability of these constructs. Based on these results, we observed some improvements to ActRIIB-Fc homodimers by replacing the linkers with a more rigid one, but even these linkers can be further optimized to prevent sequence-related clipping and increase folding rate.

Dedication

To my parents

Acknowledgments

This thesis would not have been possible without knowledge and guidance of Brantley Herrin (thesis director) and Rose Grenha (thesis mentor). Also, Roselyne Castonguay (Director of protein purification and characterization groups at Acceleron Pharma) for allowing me to conduct my research in her groups. The help her team along with Louis Liu provided in training me on different characterization techniques was invaluable. A special mention goes to Matt Lepardo for assisting with proofreading and . Molecular and Cell Biology group at Acceleron Pharma for allotting time and space to conduct my studies. All my co-worker for all their encouragement and support through-out this process. Last, but not least, Acceleron Pharma for their dedication and support for personal growth of their employees.

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Chapter I.

Introduction

Fc fusion proteins are one of the most successful biologic therapeutic formats with 11 FDA approved drugs in its class that have shown efficacy in a broad range of diseases (Jafari et al., 2017). An Fc fusion protein is constructed by attaching the CH2-CH3 domains comprising the Fc region of human IgG isotypes to the extracellular domain (ECD) of a protein of interest by a linker. Most Fc fusions are homodimers with the same ECD expressed on both arms of the disulfide-linked, dimeric IgG-Fc. The ECD domain on the fusion protein can either stimulate a receptor on the cell surface or competitively bind a ligand to interfere with receptor-ligand interactions (Czajkowsky et al., 2012). The advantages of Fc fusion proteins as therapeutics include ease of purification using Protein A, which binds to the IgG-Fc, and increased serum half-life due to recycling from endosomes mediated by FcRn (Roopenian et al., 2007).

Aggregation is a major issue with recombinant proteins during manufacturing, which compromises the quality, safety, and efficacy of the drug (Vazquex-Rey et al., 2011). A high level of aggregation impurities in therapeutics can cause immunogenicity in patients by inducing an immune response toward the drug, which can result in faster clearance or neutralization leading to limited efficacy or allergic symptoms, such as anaphylaxis; hence, posing serious safety risk (Baker et al., 2010). Aggregated products are caused by misfolded proteins, which can be removed during purification but at a reduction in yields. Aggregation can also be ameliorated by modification of hydrophobic residues in the polypeptide, which are known as aggregation “hot-spots”. Fc fusion

proteins often have more aggregation issues than monoclonal antibodies, because the ECDs used to make the fusion are typically derived from membrane-bound receptors, and the unnatural pairing of an ECD with an IgG-Fc can lead to protein folding issues. Therefore, it is necessary to optimize the cellular/molecular parameters that affect expression and quality of the protein, including but not limited to selecting a suitable linker, to increase the yield of correctly folded protein.

Protein Synthesis and Folding

Synthesis of a secreted polypeptide begins in the cytosol before it is translocated to the endoplasmic reticulum (ER) for folding into a functionally active protein. The amino acid sequence of a polypeptide contains the information essential for the folding of a partially linear polypeptide into a functional three-dimensional native conformation (Anfinsen et al., 1972). Initiation of protein folding occurs in multiple ways including hydrophobic collapse, which occurs spontaneously via interactions between nonpolar sidechains of a liner polypeptide. These interactions lead to a more compact structure containing a high degree of alpha helices and beta sheets resulting in a partially folded molecule with some secondary structure (Brylinski et al., 2006). Other protein folding mechanisms include the formation of disulfide bonds between cysteines and the formation of a secondary structure via non-covalent intramolecular interactions laying the foundation for subsequent folding into tertiary and quaternary structures (Kim et al., 2013).

The process of folding occurs in several steps where the polypeptide undergoes multiple intermediate structures before folding into a biologically functional protein (Ellis et al., 2006). These transitional structures are highly prone to misfolding due to stressors

within the cellular environment leading to a thermodynamically unfavorable non-native confirmation. Therefore, the protein unfolds and refolds until a more favorable native confirmation is achieved (Ellis et al., 2006; Dill et al., 2012). In its native form, proteins have a hydrophobic core with hydrophobic residues buried within. In contrast, misfolded, partially unfolded, and altered proteins have exposed hydrophobic surface residues, and interactions between these exposed residues may give rise to aggregation (Khan et al., 2013). Covalent and non-covalent interactions allow for misfolded monomers to assemble into a higher order structure with decreased solubility, leading to aggregation (Siddiqi et al., 2017). It is a sequential process starting with individual native, or misfolded, monomers interacting with other monomers to form an oligomer followed by the formation of mature fibrils before arranging into insoluble aggregates (Wang et al., 2018). It has also been shown that one species can influence the aggregation of another species, or two different species can co-aggregate during the process of folding (Rajan et al., 2001)

Factors Affecting Protein Folding

Many factors have the potential for generating partially folded or folding intermediates leading to protein aggregation. These factors can affect different stages of protein production in vitro. Polypeptide sequences can contain residues sensitive to clipping, oxidation, deamidation, and isomerization along with free cystines with potential for disulfide scrambling (Chiti et al., 2006). Mutation in these sequences can also hinder the function of sequence-specific chaperons that aide with folding (Chiti, et al., 2006). Proteins with hydrophobic patches and charged residues on its surface also have the potential for misfolding as discussed later in this publication. In construct

design, factors such as orientation of the active domains and the linkers that separate these domains have a role in folding and aggregation (Blamowska et al., 2009). During expression and purification processes, parameters such as freeze-thawing and other temperature fluctuations can weaken or break non-covalent bonds between amino acids (Cao et al., 2003), physical stress such as filtration, shearing and stirring can also weaken these interactions (Mahler et al., 2015), solvent and storage conditions such as very acidic and very basic pH of the solution can change protonation of the amino acid causing an increase or decrease non-covalent interactions (Elgersma et al., 2014). All these factors initiate different pathways of aggregation in solution.

Aggregation Pathways Affecting Protein Folding

There are several mechanistic pathways involving native or misfolded/altered confirmations that can cause protein aggregation. These pathways are not independent of each other and multiple can occur simultaneously (Philo et al., 2009). Examples of these include reversible association of monomeric proteins, aggregate formation by conformationally altered proteins, aggregation of chemically-modified product, nucleation-controlled aggregation, surface-contact driven aggregates, 3-D swapping and amyloid cascade hypothesis (Strand et al., 2012; Zaman et al., 2019; Philo et al., 2009).

Mechanism 1. Reversible Association of Monomeric Protein

Reversible association of monomeric proteins pathway addresses the self-association feature of the native monomer to form oligomer. These are weak and reversible interactions that occur via self-complementary patches on the surface of monomers, and these monomers can be arranged in side-side and end to end

conformation (Strand et al., 2012). With increased concentration of the protein, there is an increase in number of monomers that can self-associate and form a larger oligomer, which over time become aggregates that are irreversible (Philo et al., 2009).

Mechanism 2: Aggregate Formation by Conformationally Altered Protein

Aggregate formation by conformationally altered protein is very similar to reversible association of monomeric protein in which interaction of monomers leads to formation of oligomers. The main difference between the two pathways is conformational change. Initiation of this pathway occurs when native conformation undergoes a conformational change, due to which the interaction between the monomers becomes much stronger. Conformational change is triggered mostly by stress, such as heat or shear (Philo et al., 2009).

Mechanism 3: Aggregation of Chemically-Modified Product

The third pathway, aggregation of chemically-modified product, is similar to the second mechanism in which conformational change causes aggregation. This change in conformation is due to differences in covalent structure, which is caused by chemical degradation, such as oxidation of methionine. These chemical changes can create new self-complementary patches or alter electrostatic interactions between monomers, allowing for the monomer to interact strongly (Hermeling et al., 2006; Philo et al., 2009).

Mechanism 4: Nucleation Controlled Aggregation

The nucleation mechanism of aggregation builds on the first mechanism, where native monomers with low tendency to interact with each other form an oligomer. Upon

reaching a certain size, the oligomer becomes what is known as the “critical nucleus”. Once the critical nucleus is formed, any addition of monomers becomes favorable, leading to formation of larger species allowing for aggregation to forms at a higher rate and become visible in solution as precipitates. This mechanism is known as primary nucleation or “homogenous nucleation”. An alternative nucleation mechanism, called secondary nucleation or “heterogenous nucleation”, can also occur where the critical nucleus is composed of impurities and contamination present in culture (Philo et al., 2009; Siddiqi et al., 2017).

Mechanism 5: Surface-induced Aggregation

Surface-induced aggregation occurs when the native monomers interact with the surface of the container or air-liquid interfaces via electrostatic and hydrophobic interactions, respectively. After the initial interaction with different surfaces, the monomer goes through conformational change and aggregates. It is also possible that these conformationally-altered monomers are released back in solution and aggregate when they interact with the surface of the container (Siddiqi et al., 2017; Wang et al., 2010).

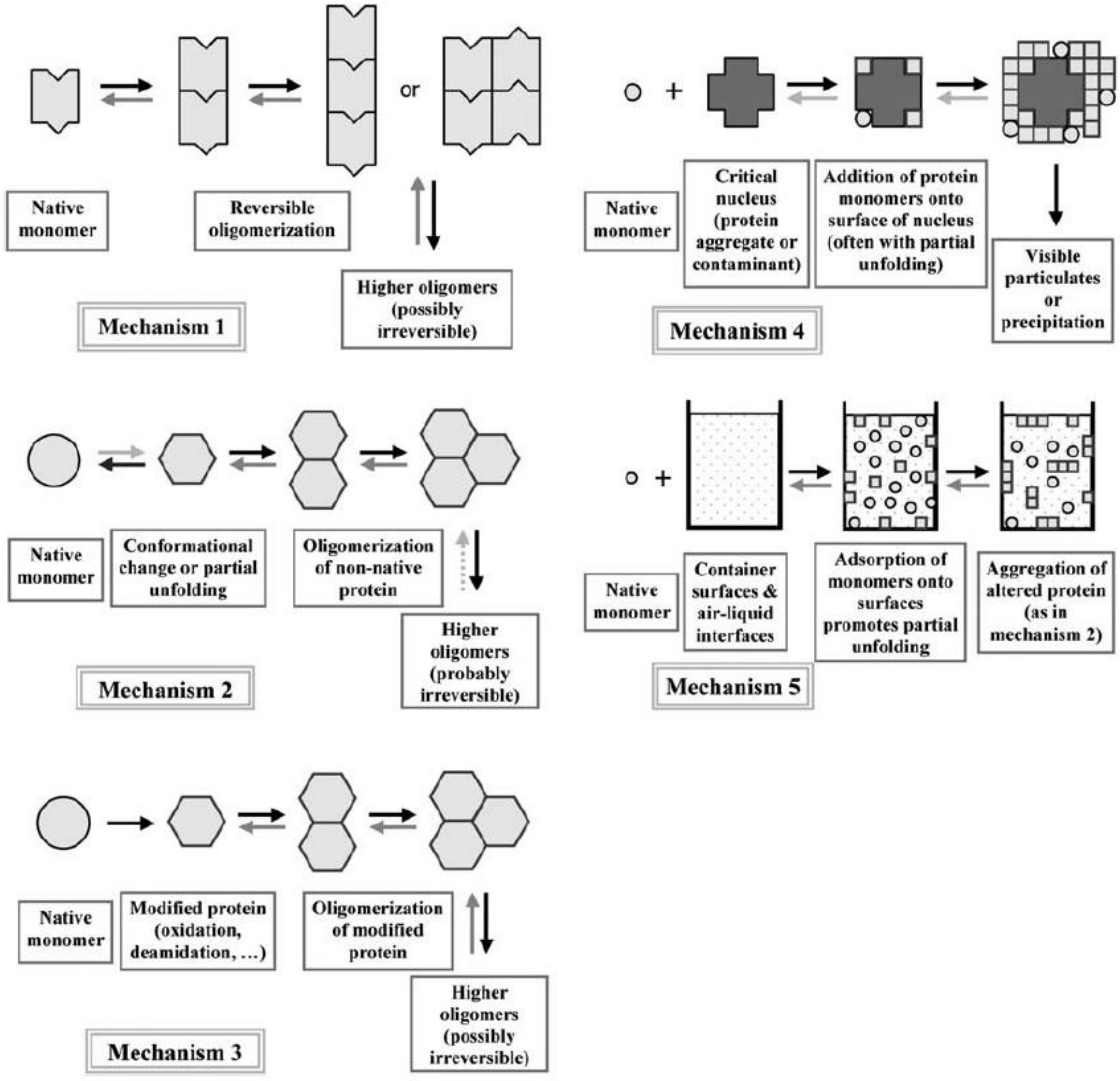

Figure 1. Mechanisms of Aggregation

Mechanisms of aggregation includes Reversible association of monomeric protein, aggregation formation by conformationally altered protein, aggregation of chemically-modified products, nucleation controlled aggregation and surface-induced aggregation. From "Mechanism of Protein Aggregation" by John S. Philo, 2009, Current Pharmaceutical Biotechnology, 10, p 348. Copyright 2009 Bentham Science Publishers Ltd.

Mechanism 6: 3D-swapping

3D-swapping or domain swapping is an aggregation mechanism involving two protein domains connected with a hinge loop that can cross interact and form aggregates. These can be found in closed or open monomers. The closed monomer complex forms when the “swapped” domain interacts with its’ own “core” domain. Open monomer complex occurs when the “swapped” domains is available to interact with “core” domain of other open monomers by self-complementation. The open monomer complex can be arranged in a 3D domain swapped dimer, involving two open monomers, or open-ended runaway swap, involving multiple monomers and can lead to arrangements that form higher-order aggregates in culture (Bennett et al., 2006).

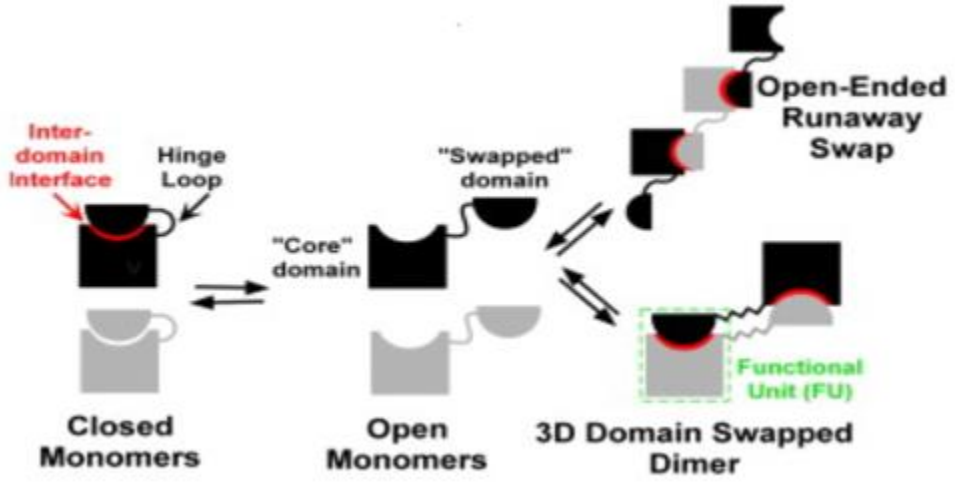

Figure 2: Mechanism of Aggregation Illustrating 3D-swapping.

Illustrates mechanism of aggregation involving interaction of a domain of a monomer with the second domain of a different monomer. Taken from “Deposition Diseases and 3D Domain Swapping” by Melanie J. Bennett, 2006, Structure,14 (5), p811. Copyrights 2006 Elsevier Ltd.

Linker Properties and its Influence on Fc-Fusion Proteins

The linker segment that connects the extracellular domain (ECD) of a receptor to the antibody constant region is an important design element of an engineered Fc-fusion protein. It not only controls the overall flexibility of the structure of an Fc-fusion protein, but it also can influence the interaction between the protein domains by varying the rigidity and distance of the span in fusing them together (Rosmalen et al., 2017). This influence of the linker is largely dictated by the amino acid sequence, allowing for local relaxation of peptide bond angles, and these local changes in the bond angles can translate into significant changes in the overall structure of the proteins, contributing to the structure's overall flexibility (Wriggers et al., 2014). Linkers are useful in separating the ECD from the Fc domain of the monomer and allowing for their independent folding, but they can also give the two ECDs of the dimerized molecule the distance and flexibility needed to bind ligands, which can influence the avidity of the interaction (Klein et al., 2014). Hence, linkers directly influence the functional properties of the fusion protein.

Characteristics of a Linker

Linkers are classified as either flexible, typically glycine-rich sequences, or rigid, including proline-rich sequences and sequences encoding alpha-helical secondary structures (Chen et al., 2013), which can be naturally occurring or synthetic. For an Fc dimer, linker selection depends on whether the two arms need the flexibility to come together or the rigidity to separate the two ECDs. Therefore, factors such as length, hydrophobicity, amino acids residues and secondary structure found in a natural linker may play an important role in linker selection (Chen et al., 2013).

George and Heringa et al. showed that there is an inverse relationship between linker length and hydrophobicity, whereby a longer linker might be less hydrophobic and more accessible to solvents (George and Heringa et al., 2002). A study conducted by Robinson et. al., demonstrated the impact of varying linker length and residue composition translated into a six-fold increase in the expression of the protein (Robinson et al., 1998). The length of the linker not only affected the function and stability but also the folding and unfolding kinetics of the fusion protein, where increased rates were seen with shorter linkers (Aria et al., 2001, Nagi et al., 1997). An increased prevalence of amino acids with polar uncharged and charged side chains has been observed in natural linkers with preference for proline, threonine, and glutamine along with serine and glycine (Argos et al., 1990). In particular, proline is a unique amino acid with a cyclic ring side chain which restricts the conformation by introducing a kink in the structure and adding rigidity to the linker (Williamson et al., 1994). It also lacks amide hydrogen in the peptide backbone which prevents formation of hydrogen bonds with other amino acids, thereby hindering the formation of secondary structures and reducing any aberrant interaction between the linker and the protein domains. As a result, the inclusion of proline residues might be useful in increasing the stiffness and structural independence of the linkers (Radford et al.,1987). Threonine, serine and glycine are favorable in providing flexibility due to small side chains and maintain stability of the linker structure in the solvent through formation of hydrogen bonds with water (Argos et al., 1990).

Linkers can also adopt secondary structures in the form of α-helix, β-sheets, β-turns and Ω-loops to ensure interaction and functionality of the domains (Chen et. al., 2014). Most of the linkers observed in nature are either helical or coiled/bent structures

with slight variations depending on the length (Aria et al., 2001). Helical structures are formed when intra-segment hydrogen bonds between amino hydrogen and carboxyl oxygen atoms in the backbone forming a closely packed structure. These interactions give the linker the rigidity and stability needed to separate the two functional domains and allow for proper folding with minimal interaction with the linker or with each other. Hence, the helical secondary structure is found in most naturally occurring linkers (Aurora et. al., 1998). The non-helical linkers are lacking the rigidity of the alpha-helix, which can be compensated for by addition of proline for the required stiffness and reduction in inter-domain interference (Willaimson et al., 1994).

Type of Linkers

Flexible linkers are mostly widely used in the biopharmaceutical industry, and these comprise of small polar (glycine) and non-polar (serine and threonine) repeats. Glycine residues in GS linker have been shown to influence the flexibility of the linker (Yan et al., 1996). There is an inverse relationship between the number of glycine residues and the rigidity of the linker, where an increase in rigidity is paired with a decrease in glycine content (Rosmalen et al., 2017). Serine and threonine residues add to the stability of the linker by forming hydrogen bonds with the aqueous environment, thereby preventing any inter-molecular interactions between the linker and the protein domain potentially leading to reduced interference with the folding and function of the domains (Yan et al., 1996). Most flexible linkers are (G4S)n repeats, but other residues, such as threonine, alanine, lysine and glutamic acid, can also be incorporated to maintain flexibility (Chichili et al, 2013). Flexible linkers may be useful in connecting protein domains that require a certain degree of movement to interact, either with each other or

with the ligand. Rosmalen et al., also reports that flexible linkers are only advantageous when covering a short distance of less than 60Å (Rosmalen et al., 2017) For a wider separation, flexible linkers may not provide adequate separation of the two ligand binding sites on the Fc-fusion/antibody. The length on flexible linkers can vary to allow spatial distance for proper folding or optimal biological activity of the fusion proteins, but lack of rigidity could potentially affect the expression and function of certain proteins (Chichili et al, 2013). This is possibly due to inefficient separation of the two protein domains or interference with each other. Rigid linkers would be a more appropriate choice in this case, particularly α-helical linkers, which are stiffer and provide the distance and stretch needed for each protein domain to fold and function independently, leading to an increase in thermal stability (Radford et al., 1987).

Function and Influence of the Linkers on Folding and Expression

Functional application of a linker on the expression and function of an Fc fusion protein is well documented in literature. Linkers form covalent linkages with the proteins and influence their biological activity, PK profiles and yields in culture, but the exact mechanism is still unclear (Chen et al., 2014). However, it has been hypothesized that the length and the amino acid composition of the linker adds to its stability, which in turn ensures proper folding and enhances the stability of the newly translated polypeptide and consequently, increase the expression (Amet et al., 2011)

As discussed earlier, aggregates are composed of misfolded proteins that possess biological activity different from the properly folded monomer resulting in decreased yield, altered therapeutic efficacy and unwanted immune responses, which can lead to formation of anti-drug antibodies in vivo (Ratanji et al., 2014). Among other factors, use

of an incorrect linker (too short or flexible) to separate the two protein domains may promote misfolding. Huang et al. used hydrogen-deuterium exchange mass spectrometry (HDX-MS) and spatial aggregation propensity (SAP) to demonstrate that use of an unstructured linker can create a hot patch in the CH2 domain of the Fc region. They characterized aggregation propensity of FcP1 fusion proteins by analyzing the CH2 domain connected to the antigen binding domain by a flexible, unstructured linker. HDX-MS measures the exchange of amide hydrogens, which are exposed during the unfolding event, with a deuterium molecule and the readout is the speed at which deuterium uptake occurs. Faster uptake indicates higher solvent accessibility and/or hydrogen bond formation. The data collected in this study shows significant deuterium uptake and high SAP reading in linker region and the CH2 domain indicating stress induced unfolding leads to unstable CH2 domain with exposed hydrophobic residues, which would otherwise be buried with amide-amide interaction instead of amide-water. This suggests increased aggregation propensity of region 145-163 in the CH2 domain is likely caused by the neighboring unstructured linker region, which facilitated the exposure of the hydrophobic residues in region 145–162 under stress conditions and initiated the self-association (Huang et al., 2016, Eberhardt et al., 2011).

For glycine-rich linkers, another aspect that needs to be considered is the post-translation modifications (PTMs) discussed in studies published by Spahr et al. (2014), Spencer et al. (2013), and Tyshchuk et al. (2017). The serine residue in the GSG motif is the preferential site for the xylose-based glycosaminoglycan (GAG) core in CHO cells, which leads to an increase in o-xylosylation in fusion proteins containing the GS linker. Any such increase in glycosylation may produce a molecule that is highly prone to

aggregation and may potentially have a negative impact on activity, stability, and safety of the molecule (Spahr et al., 2014, Spencer et al., 2013). Serine residues are prone to phosphorylation as reported by Tyshchuk et al. They detected a mass of +79Da variant by MassSpec and collision-induced dissociation (CID) which corresponded to phosphorylation of the last Serine residue at the C-terminal of the linker followed by the Fc. The phosphorylation event is dependent on the flanking region of the linker where the +2 position is dominated by glutamic acid (Tyshchuk et al., 2017). Phosphorylation is an important event in many signaling pathway and cellular processes.

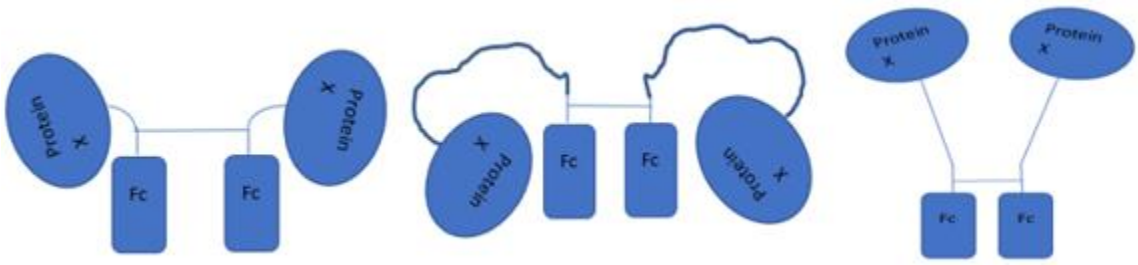

Figure 3: Expected Confirmation for Protein with Different Linkers

Illustrates structural difference in GGG (control linker 1 left), (G4S)4 (control linker 2 center) and hIgA1hinge/(AP)x11 linkers (test linkers right)

This study was designed to assess changes in expression, yield, and stability of ActRIIB with rigid linkers compare to the controls (Figure 3) by examining growth and viability of the cultures as well as assess aggregation, low molecular weight species and stability of protein during purification. We also compared binding, inhibition, and thermal stability of these molecules by looking at parameters such as binding affinity (KD) and inhibition by comparing IC50. Thermal stability of the molecules can be

evaluated by measuring the melting temperature (Tm), aggregation temperature (Tagg), sizing and polydispersity using intrinsic fluorescence of residues, static light scattering (SLS), dynamic light scattering (DLS) and differential scanning calorimeter (DSC). These are different methods for measuring melting temperature and aggregation by utilizing intrinsic fluorescence of aromatic amino acid with and without dyes, changes in light scattering due to increase in size or changes in heat absorption as the protein denatures using Differential Scanning Colorimetry (DSC). The insight gained from this study can contribute towards selecting linkers of appropriate length and rigidity for other molecules in the future.

Chapter II.

Material and Methods

This section details the materials and methods used to conduct the following study. Briefly, two test ActRIIB-linker constructs were cloned from synthesized fragments and expressed, along with control linkers, in ExpiCHO cells. These molecules were purified using Protein A chromatography followed by preparative SEC chromatography. The quality of the molecules was analyzed using analytical SEC, SDS-PAGE and western blots after each purification step with an additional characterization step after preparative SEC to assess difference in binding of the molecules using a human anti-ActRIIB antibody column. Additional assays were performed to evaluate changes in binding kinetics, activity, and thermal stability of the homodimer introduced by the linkers.

Production of the Fc-Fusion

Fragments consisting of ActRIIB extracellular domain (ECD) with humanIgA1 hinge and ActRIIB (AP)x11 linkers were synthesized by Twist Biosciences (South San Francisco, CA) shown in Table 1. Subcloning of these fragments into expression vector containing the Fc region of the IgG was performed using the protocol provided by NEBuilder® HiFi DNA Assembly cloning kit (New England Biolabs, Ipswich, MA), prior to which the vector and the fragments were digested for 1 hr. at 37 ℃ with NheI and AgeI restriction enzymes (New England Biolabs, Ipswich, MA). Gel electrophoresis

was performed to isolate the vector and the insert bands using a 0.8% agarose gel prepared from certified molecular biology agarose (Bio-Rad, Hercules, CA) made in 1xTAE buffer (Invitrogen, Carlsbad, CA) and ethidium bromide (Bio-Rad Hercules, CA) was added for visualization. Gel purification was performed to isolate DNA using protocol provided by Qiagen gel purification kit (Qiagen, Hilden, Germany). Transformation was performed using 1µL ligated product (diluted 1:4x) in 20 µL of ElectroMax DH5alpha-E Competent Cells (Invitrogen, Carlsbad, CA) using Bio-Rad MicroPulser Electroporation (Bio-Rad, Hercules, CA). Transformed bacteria was plated on agar plates containing 100 µg/mL ampicillin purchased from Teknova Inc. (Hollister, CA) and incubated overnight at 37 ℃. Colonies were selected for miniprep, and DNA was extracted using the protocol provided by Qiagen Miniprep Kit (Qiagen, Hilden, Germany). The clones were confirmed to have the correct insert by restriction enzyme digestion and sequencing. The clone with the insert was streaked out on agar plates with ampicillin and incubated at 37 ℃ overnight. The next morning a single colony was inoculated in LB broth (ThermoFisher Scientific, Grand Island, NY) containing 10% of Ampicillin (Acceleron Pharma, Cambridge, MA). DNA was extracted by maxiprep following the protocol provide by ZymoPure II (Zymo Research, Irvine, CA). The open reading frame of the DNA was sequenced by Wyzerbio (Boston, MA) to confirm the presence of correct insert consisting of ActRIIB ECD-linker-Fc domains. The control constructs were previously cloned in-house.

Table 1. Sequences of the ECD with Linkers

Molecules	Protein sequence	DNA sequence
ActRIIB Control 1	ActRIIB-GGG	ActRIIB- GGTGGTGGA
ActRIIB Control 2	ActRIIB-GGGGSGGGGSGGGGSGGGGS	ActRIIB-GGTGGTGGAGGTTCTGGAGGTGGAGGAAGTGGTGGAGGTGGTTCTGGAGGTGGTGGAAGT
ActRIIB-hIgA1hinge	ActRIIB-GPSTPPTPSPSTPPTPSPS	ActRIIB-GGTCCATCTACCCCTCCAACACCTTCTCCTTCCACCCCTCCTACCCCCTCCCCTAGC
ActRIIB-(AP)x11	ActRIIB-GAPAPAPAPAPAPAPAPAPAPAP	ActRIIB-GGTGCCCCTGCTCCTGCTCCTGCTCCAGCTCCTGCTCCCGCTCCTGCCCCTGCCCCCGCCCCTGCCCCT

Protein and DNA sequences of the linkers

For the first round of expression, ActRIIB hIgA1hinge, ActRIIB (AP)x11, and controls ActRIIB GGG, ActRIIB (G4S)4 were expressed in 1 L ExpiCHO culture in 3 L Corning flask (Corning, Tewksbury, MA) following the max titer protocol suggested by the manufacturer (ThermoFisher Scientific, Grand Island, NY). Cells at the density of 6x106 cells/mL and with viability above 99% were transfected using 0.7 µg/mL of DNA complexed with 640 µL of ExpiFectamine, cationic lipid-based transfection reagent provided in the ExpiCHO kit. The cultures were incubated overnight at 37 ℃ with 5% CO2 and a shake speed of 80 rpm in a Bellco incubator (Bellco, Vineland, NJ). Feed and Enhancer were added to the cultures on day 1 and the cultures were temperature shifted

to 32 ℃ at 5% CO2. Cultures remained in these conditions until day 12 with an additional feed on day 5 and periodic cell counts measurements to ensure culture quality. These were harvested by centrifugation at 4000 rpm for 20 mins at 4 ℃ and filtered through 0.2 µm pore size filter (Corning, Tewksbury MA). Each culture was divided into four 250 mL aliquots and kept at -20 ℃.

Different culture parameters, such as variable cell density (VCD), viability, and protein titer, were gathered for each culture. The VCD and viability of each culture was measured using a Vi-CELL™ XR Cell Viability Analyzer (Beckman Coulter, Indianapolis, IN). The titer measurement was taken by a series of 8-step, 5-fold dilutions in PBS in a 96-well plate using the Octet QKe (ForteBIO Inc., Torrance, CA). A biosensor coated with protein A (ForteBIO Inc. Torrance, CA) was used to capture the Fc-fusion molecules by binding to the Fc region. The difference in signal between the baseline, determined by PBS only wells, and the sample was tested and analyzed using the Octet QKe. A titer of the samples, measured in µg/mL, was determined by utilizing the binding rates of the pre-established standard curve with known concentration.

Pre-Equilibration of FASTPURE MIDI Spin columns (G-Biosciences, Louis, MO) were sanitized using 0.1 N NaOH (VWR, Radnor, PA) and rinsed with distilled water before use. MAb Select Sure Resin (Cytiva, Westborough, MA)) was prepared by rinsing twice with 30 mL of distilled water before sanitization with 20 mL of 0.1 NaOH for 30 min at room temperature, followed by a conditioning step using 30 mL x2 0.1 M Glycine pH 3.0 and equilibrated with 30 mL x 3 TBS pH 8.0. All steps were centrifuged in a Sorvall RC 3BFT Centrifuge (ThermoScientific, Rockland, MD) at 1500 rpm for 10 mins at 4 ℃.

A 1.5 mL of equilibrated MAb Select Sure resin was used to pack FASTPURE MIDI Spin column. The spin speed for each step was 1500 rpm for 10 mins at 4 ℃ in centrifuge (ThermoFisher, Scientific Waltham, MA). A 60 mL volume of GGG and (G4S)4 linkers, 150 mL of hIgA1 and 360 mL of (AP)x11 from 1 L cultures were purified in round 1 (Table 2) to retrieve enough material for characterization. The flow-through consisting of all unbound material was collected and reapplied to ensure maximal recovery. Columns were washed with TBS pH 8.0 after the binding of molecule to the column. Elution was performed using 7 mL of 0.1 M glycine at pH 3.5 and 6 mL of 0.1 M glycine at pH 3.0. Neutralization was performed using 10% 1 M Tris p H8.0. The protein was concentrated using Amicon® Ultra 15 mL Centrifugal Filters 30 kDa NMWL (Millipore Sigma, St. Louis, MO) and was equilibrated with PBS at 4000 rpm for 5 mins at 4 ℃. Elution collected in glycine pH 3.5 and neutralized with1 M Tris were combined for each sample after characterization and concentrated by applying the sample to equilibrated Amicon® Ultra 15 mL Centrifugal Filters and centrifuged at 4000 rpm at 4 ℃ for multiple 5 min intervals until the final volume was 7-10 mL.

Preparative SEC was used as a second purification column using size-based separation method. Preparative SEC was performed using Superdex® 200 Increase 26/600 (Cytiva, Marlborough, MA) packed with a dextran-agarose composite matrix of 34 µm particle size and flow rate of 2.6 mL/min. The column was sanitized using 0.1N NaOH for over 10 hours to potentially remove impurities and endotoxin and rinsed with PBS. To maintain the resolution of the column, about 10% of the column volume in terms of product volume is loaded on the column, which would be a sample volume of 7-10 mL in PBS were injected into the column. Elution peaks were collected in 15-40 mL

aliquots of PBS. These were kept at 4 ℃until they were concentrated using Amicon® Ultra 15 mL Centrifugal Filters (Milliapore Sigma, Burlington MA) with a 30kDA cut-off and quantified by absorbance at 280 nm a NanoDrop 2000 spectrophotometer (ThermoScientific, Rockland, MD) using molecular weight and theoretical extinction coefficients of each molecule and stored at -80 ℃.

A second round of expression was warranted based on the low molecular weight species seen for hIgA1 hinge and (AP)x11 from round 1 of expression and purification to check for reproducibility. All four molecules were re-expressed in a small culture volume of 120 ml ExpiCHO in 500 mL Corning flask (Corning, Tewksbury MA) following the protocol described above. Cell density on the day of transfection was 6x106 cells/mL and viability of above 99%. A 0.7 µg/mL of DNA was complexed with ExpiFectamine before transfection. The cultures were incubated at 37 ℃ at 5% CO2 overnight at 120 rpm in BellCo Biotech incubator (BellCo Biotech, Vineland, NJ). Feed and Enhancer were added to the cultures on day 1 and the cultures was temperature shifted to 32 ℃ at 5% CO2 and remained in these conditions until day 14 with an additional feed on day 5 and periodic cell counts measurements to ensure culture quality. Cultures were harvested by centrifugation at 4000 rpm for 20 mins at 4 ℃ and filtered through 0.2 µm pore filter (Corning, Tewksbury, MA). Cell counts were measured on different days to monitor the cultures and the titer was taken on day 14 using the Octet QKe. The entire 120 mL culture of the test linkers was purified using MAb Protein A column and a different preparative SEC column, Sepax SRT-10 SEC-300 (Sepax Technology, Newark, DE) was used. Sepax SRT-10 Sec-300 is packed with spherical silica beads of pore size 10 µm and flow rate of 7-10 mL/min was used for round 2 of cultures. The column was washed with

multiple rounds of ethanol and PBS + 0.2%Tween20 and equilibrated in PBS before a 3.5 mL aliquot of the sample was injected. Peak containing the molecule of interest was collected in two 3.5-5 mL aliquots from preparative SEC and characterized the same way as the 1 L cultures. The only alteration is the storage duration of these lots was one night at 4°C between protein A and preparative SEC chromatography, while the 1 L was stored at 4 °C for a week and frozen at 80°C between the two purification steps.

Table 2. Culture and Purification Volumes

Molecules	Culture volume	Volume purified
GGG	1 L	60 mL
(G4S)4	1 L	60 mL
hIgA1hinge	1 L	150 mL
(AP)x11	1 L	360 mL
GGG	120 mL	Not purified
(G4S)4	120 mL	Not purified
hIgA1hinge	120 mL	100 mL
(AP)x11	120 mL	100 mL

The volume expressed and the volume purified for each culture is listed in this table

Assay for Characterizing the Fc-Fusions

The quality of these proteins was assessed on a Zenix-C SEC-300 Analytical SEC column purchased from Sepax Technology (Newark, DE) A 50 µg of purified protein was diluted in 110 µL of PBS and injected in Alliance HPLC System (Waters Corp. Milford, MA). The species ratio in solution were analyzed using Empower3 software licensed from Waters Corp (Milford, MA).

Additional aliquots of 1 µg and 5 µg were prepared for SDS-PAGE gel and western blots for each sample using 1x NuPAGE™ LDS Sample Buffer (4X) with and without 10% beta-mercaptoethanol. Reduced protein was heated at 90 ℃ and non-reduced was heated at 70 ℃ for 5 mins before loading on the gel. NuPAGE™ 4 to 12%, Bis-Tris, 1.0 mm, Mini Protein Gel, 12-well (ThermoFisher Scientific, Grand Island, NY) was used to analyze different species in each sample using 1x NuPAGE™ MES SDS Running Buffer (20X) at 200 v for 35 mins at room temperature. SDS-PAGE gels were stained using Instantblue Coomassie Stain (Abcam, Toronto, ON). For western blots, Invitrogen™Nitrocellulose/Filter Paper Sandwich, 0.2 µm (LC2000 ThermoFisher Scientific, Grand Island, NY) was soaked in NuPAGE™ Transfer Buffer (20X) before fast transfer was performed using Thermo Scientific™ Pierce™ G2 Fast Blotter (ThermoFisher Scientific, Grand Island, NY). A 2x BSA diluted in TBST with 0.1% Tween20 (Sigma, St Louis, MO) was used to block the membrane at 4 ℃ overnight. Human Activin RIIB Antibody (AF339 or BAF339 R&D systems) at 1:2000 dilution in 10ml volume was incubated at 4 ℃ for 4 hours on shaker followed by Horseradish Peroxidase Avidin D from Vector Lab Inc. (A-2004 Burlingame, CA) at 1:10000 in 10 mL for 1 hour at room temperature. A second blot was incubated with mouse anti-human

IgG Fc-HRP from SouthernBiotech (Birmingham, AL) to detect proteins with human Fc. The antibody used was at a dilution of 1:10000 and the blot was incubated for 4 hours at 4 ℃. All the washes were 10 mL TBST. Pierce™ ECL Western Blotting Substrate from ThermoFisher Scientific (Grand Island, NY) was used for developing the blots. The blots were developed on ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA).

An in-house proprietary anti-ActRIIB affinity column was used to distinguish the amount of unbound material in solution. A 50 µg of purified protein was diluted in 110 µl of PBS and injected in Alliance HPLC System (Waters Corp. Milford, MA) for this test.

Surface Plasmon Resonance (SPR) measure binding affinity of the proteins by measuring changes in refractive index proportion to changes in mass with each binding event. SPR is performed using CM5 chips composted of Carboxymethylated dextran matrix coated with anti-hFc antibody (Sigma, St. Louis, MO) on Biacore T100 (GE Healthcare Bio-Science, Uppsala, Sweden). Purified ActRIIB-GGG, ActRIIB-(G4S)4, ActRIIB-hIgA1hinge and ActRIIB-(AP)x11 was immobilized on the chip at flow rate of 10 µl/min at a concentration of 10 µg/mL. The analytes comprised of GDF 11, Activin A and BMP 9 produced at Acceleron Pharma (Cambridge, MA). The starting concentration for these analytes are listed in Table 2. These concentrations were determined based on experimental evidence from historical data for ActRIIB-GGG control. The samples were diluted in a series of 7-step 3-fold dilutions in running buffer (HBS-1xEP + 0.5 mg/mL BSA + 350n M NaCl) flown over the ligands at a flow rate of 10 µl/min, association time of 300 s and dissociation of 600 s. A 10 nM glycine pH 1.7 was used as regeneration buffer. Affinity of the protein is determined by calculating its equilibrium dissociation constant, KD, which is a ratio of

Equation 1: KD= Koff/Kon= [A]+[B]/ [AB]

Reporter Gene Assay (RGA) measures inhibition of ligands by ActRIIB proteins. The assay requires the use of A204 cells, maintained in McCoy's Medium (Gibco, Waltham, MA) with 10% Hyclone FBS (VWR, Radnor, PA), and T98G cells, maintained in EMEM Medium (ATCC, Manassas, VA) with 10% Hyclone FBS (VWR, Radnor, PA). The cells were seeded at the density of 2 × 105 cells/mL and 1.4 × 105 cells/mL in 500 µl culture media with 10% FBS per well, respectively, then were plated in 48-well tissue culture plates (Corning, Tewksbury, MA). A204 cell line was transfected with CAGA12 and T98G was transfected with BRE reporter constructs in 500 µl assay media with 0.1% BSA. Both cell lines were transfected with a second reporter construct containing renilla luciferase gene acting as a transfection control. ActRIIB-hIgA1hinge and ActRIIB-(AP)x11 along with the controls ActRIIB-GGG and ActRIIB-(G4S)4 were diluted in an 8-step 3-fold dilution series starting at concentration listed in Table 3 Addition of equal volume of the ligand altered the concentration of the sample in well to half of its starting concentration. Plate containing the sample and ligand is incubated at 37 ℃ with 5.0% CO2 for 30 mins before adding the mixture on the cells. The cells were kept at 37 ℃ with 5% CO2 for 6 hours for GDF 11 and Activin A, and overnight incubation for BMP 9. Cell lysates were prepared using the lysis buffer and the signal was measured using dual luciferase provided in Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) in relative luciferase units (RLU) were measured using Infinite® 200 PRO (Tecan, Männedorf, Switzerland). IC50s were calculated for each.

Table 3. Concentration of Ligands and Analytes Tested in Assays

Molecules	Biacore	Reporter Gene Assay
GGG	10 µg/mL	115.4-3846 ng/mL
(G4S)4	10 µg/mL	112.5-4082 ng/mL
hIgA1hinge	10 µg/mL	300 ng/mL-10 µg/mL
(AP)x11	10 µg/mL	300 ng/mL-10 µg/mL
GDF 11	20 mM	5 ng/mL
Activin A	20 mM	5 ng/mL
BMP 9	20 mM	600 pg/mL

The volume expressed and the volume purified for each culture is listed in this table

Thermal stability of the constructs was tested by analyzing intrinsic fluorescence of tyrosine and tryptophan residues as they get exposed during denaturation of the protein during thermal ramp of 15-95 ℃, along the use of light scattering technology measuring size of the molecule as it denatures and by measuring heat absorption using DSC. The Fc fusion proteins with different linkers were prepared in PBS at a concentration of 2 mg/mL. A volume of 9 µL was loaded in the capillary in duplicates and all the samples were measured with a thermal ramp from 25-85 ℃ using a ramp rate of 0.5 ℃/min using the UNCLE instrument (Unchained Labs, Woburn, MA). The data collected was analyzed using a method called BCM (barycentric mean), which uses full spectrum

analysis (266-473nm) to measure the fluorescence intensity. DLS was used to measure the size increase and poly dispersity of the sample as the temperature increases.

DSC was performed on the same sample set diluted to 600 ng/mL concentration in 400 µL of PBS (in-house). These were added to 96-well plate with the reference cell containing PBS (in-house) to compare heat absorption difference between the sample and control upon heating up the sample 10-95 ℃. DSC thermal study was performed using MicroCal VP-Capillary DSC (Malvern Panalytical, Malvern, UK).

Data Analysis

Empower3 was used to integrate and analyze high molecular, low molecular and monomer peaks from analytical SEC column.

Biacore T100 Control and Evaluation software was used to write the methods for the assay and to analyze the data to fit the curves to 1:1 model and to calculate Ka, Kd and KD for each curve, respectively.

Data from RGA was analyzed by employing the four-parameter model to fit the dose–response relationship between RLU and log10 of the fusion protein concentration using GraphPad Prism 9, while the relative potency is expressed as half maximal inhibition concentration (IC50) ratio of the reference standard versus the sample.

UNCLE software provided by Unchained Labs is used to analyze the thermograms for each sample while simultaneously looking at Tm and Tagg from different applications. It was also used for analyzing hydrodynamic diameter at 25 ℃ and 95 ℃.

MicroCal VP-Capillary DSC Software 2.0 to used analyze the thermograms for each sample by correcting the baseline and calculating Tm for extracellular domain and for the Fc of each fusion protein.

Chapter III.

Results

This study was designed to look at the effects of linker length and rigidity on yield, folding and aggregation of ActRIIB molecules. This was achieved by expressing and characterizing two ActRIIB homodimers with long rigid linkers of unique composition, and two controls with flexible linkers, in a series of assays. Human IgA1 hinge is a naturally occurring linker found on human IgA1 antibody consisting of proline, serine, and threonine repeats, while (AP)x11 linker is a synthetic linker comprising of 11 units of alanine and proline repeats. The controls consist of flexible linkers of varying length and amino acid composition to account for length and rigidity. The most tested of the two control linkers is ActRIIB-GGG, which has proven to be a stable molecule in general with good binding to its ligands. Different assays were used to assess whether rigid linkers improve yield and aggregation, and the effect of it on the activity, binding of the molecule, stability and spatial distance between the two arms of the homodimer. The results of this study will advance our understanding of the ActRIIB molecule in-terms of preference for length and rigidity of its linker, and the required distance between the two domains of a monomer for proper folding and the spatial distance between the two ECDs of a homodimer to enable interaction for biological activity.

The Fc-Fusion Molecules

Expression and Purification of the Fc-Fusion Molecules

Rigid linkers have shown to provide the stability needed for fusion molecules to fold properly in-turn decreasing the aggregation and increasing the correctly folded, fully functional proteins (Amet et al., 2011, Klein et. al., 2014). Based on the results from several studies also looking at linker rigidity in Fc-fusion molecule, this study was designed to mimic the same concept containing ActRIIB linked to human Fc via rigid linkers. All four cultures were expressed in 1 L and 120 mL culture volumes and initial evaluation of the molecules was performed by analyzing different cell culture parameters for the duration of the culture, including titer, cell viability, and viable cell density.

For the 1 L culture, all four cultures were prematurely harvested on day 12 rather than day 14 due to mandatory laboratory shutdown because of the COVID-19 pandemic, but still had reasonable cell counts and viability for day 12 harvest (Table 4). Viability is comparable for all four constructs while the cell count is similar for three out of four showing a slightly higher count for hIgA1 hinge of about 9 x106 cells/mL (Table 4). This data indicates the cells in all four cultures were equally healthy and grew equally well (Table 8, 9); however, both the hIgA1 hinge and (AP)x11 showed low titers (Table 4), and extensive amount of low molecular species and comparatively less of the functional homodimer after MAb purification by analytical SEC (Table 10). This indicates the cells were unable to produce these proteins as well as the controls for the 1 L culture volume and required purification of additional aliquots of condition media to retrieve enough protein for characterization.

Table 4. Culture Parameters for 1 L ExpiCHO Cultures.

Molecules	GGG	(G4S)4	hIgA1 hinge	(AP)x11
Culture volume	1 L	1 L	1 L	1 L
Days in culture	12	12	12	12
Cell counts (x106 cells/mL)	8.6	8.7	9.9	8.5
Viability (%)	90.5	89	87.7	88.6
Titer (ug/mL)	189.0	289.4	98.1	85.6

Culture health indicators for 1 L ExpiCHOs for four linkers

The poor quality, specifically the low yields and high percent of low molecular weight species, of hIgA1 hinge and (AP)x11 protein from the 1 L culture warranted a second smaller culture, which are sometimes preferred by certain molecules. The 120 mL culture was expressed in the same way following the protocol provided by ThermoFisher. The difference to be noted are the flask size (3 L vs 500 mL), shaker speed (120 rpm vs 80 rpm), potential for the use of different shaker platform within the same incubator and the day of harvest is 14, two additional days compare to the 1 L. An examination of the 120 mL cultures for all four constructs shows a cell count of about 7-8x 106 cells/mL on the day of harvest, which is typical for a well-behaved protein in culture (Table 4). The viability was measured at 81-83%. The titer is comparable for GGG, (G4S)4 and hIgA1hinge and it is about 200 µg/mL, while (AP)x11 linkers shows an almost 2-fold

increase in titer indicating that the cells are producing the target protein better than the other three cultures.

Table 5. Culture Parameters for 120 mL ExpiCHO Cultures

Molecules	GGG	(G4S)4	hIgA1 hinge	(AP)x11
Culture volume	120 mL	120 mL	120 mL	120 mL
Days in culture	14	14	14	14
Cell counts (x106 cells/mL)	7.8	7.4	7.2	7.0
Viability (%)	83.2	82.7	81.6	81.9
Titer (ug/mL)	198.4	241.5	301.0	588.7

Culture health indicators for 120 mL ExpiCHOs for four linkers

Comparison of both culture volumes show comparable titers for the control linkers and shows an expected decline in cell counts and viability with day 14 cultures compared to 12-day cultures (Table 6, 7). Since the 120 mL control cultures were not purified, the impurities in these cultures, such as aggregation and LMW species, as well as properly folded monomer were not quantified by SEC; hence, these parameters cannot be compared for the two culture volumes. Some notable differences between 1 L vs 120 mL cultures of the test linker constructs include higher titers seen in the 120 mL culture with a 3-fold increase for hIgA1 hinge and over 6-fold difference for (AP)x11compared

tor the titer seen for 1 L cultures. Analytical SEC data after MAb purification shows a 2-fold increase in aggregation for hIgA1 hinge from about 12% to 25% from 1 L to 120 mL cultures, respectively (Table 6, 7). There is an increase of about 1.5-fold seen for the (AP)x11 cultures from 10.5% to 16.6% for 1 L vs 120 mL, respectively. The presence of LMW species has decreased with smaller volume culture; with a decrease to 9% from 20% was reported for hIgA1 hinge and to 14% from 39% for hIgA1 hinge and (AP)x11 by Analytical SEC after MAb. These differences in LMW species between the two culture volumes are visible in the form of an additional band just above the 25 kDa maker (figure 4), which is only seen in the conditioned media in lane 1 and 3 of anti-Fc blot, indicating that some of the clipped species seen in 1 L is only the Fc while both culture show a band at 25 kDa that appeared to have both ActRIIB and the Fc (figure 4).

Table 6. Summary of 1 L Cultures after Protein A and PrepSEC Purification

Molecules	GGG	(G4S)4	hIgA1hinge	(AP) x11
Culture volume	1 L	1 L	1 L	1 L
MAb Yield (mg)	~8.6	~10.76	~13.15	~14.72
% HMW	16.09	18.18	12.08	10.5
% Monomer	83.57	81.07	67.64	51.3
% LMW	0.35	0.76	20.26	38.9
PrepSEC Yield (mg)	7.0	8.5	7.7	7.3 (pool 5 and 5.1)
% HMW	0.87	0.41	0.97	Pool 5: 9.02 Pool 5.1: 0
% Monomer	98.36	98.86	89.7	2 different species (9-10/10-11 mins) Pool 5: 68.04/18.26 Pool 5.1:7.59/ 83.6
% LMW	0.78	0.74	9.24	Pool 5: 4.49 Pool 5.1: 8.82
Anti-ActRIIB affinity column (% bound)	57.13	68.45	71.12	Pool 5: 43.74 Pool 5.1: 60.73
Final Yield (mg)	~6.9	~8.36	6.9	3.0/4.0

Yields and analysis of different species in 1 L cultures

Table 7. Summary of 120 mL Cultures after Protein A and PrepSEC Purification

Molecules	GGG	(G4S)4	hIgA1hinge	(AP) x11
Culture volume	120 mL	120 mL	120 mL	120 mL
MAb Yield (mg)	NA	NA	25.2	58.2
% HMW	NA	NA	24.98 (avg)	16.6 (avg)
% Monomer	NA	NA	66.03 (avg)	69.4 (avg)
% LMW	NA	NA	8.98 (avg)	13.94 (avg)
PrepSEC Yield (mg)	NA	NA	19.6	20.07
% HMW	NA	NA	0	0
% Monomer	NA	NA	Peak A5: 100 Peak A6: 58.64	Peak B5: 77.08 Peak B6: 24.91
% LMW	NA	NA	Peak A6: 41.36	Peak B5: 22.92 Peak B6: 75.09
Anti-ActRIIB affinity column (% bound)	NA	NA	76.5	73
Final Yield (mg)	NA	NA	16.02	15.75

Yields and analysis of different species in 120 mL cultures

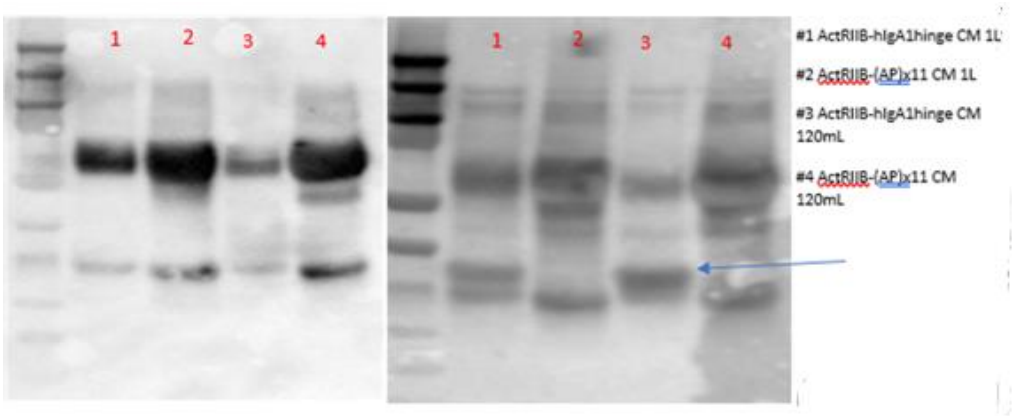

Figure 4. Characterization of CM from 1 L and 120 mL cultures by Western Blots

Culture comparison of ActRIIB hIgA1 hinge and ActRIIB (AP)x11 in 1 L vs 120 ml volume. Lane 1,2 hIgA1hinge 1 L and 120 ml, respectively. Lane3,4 (AP)x11 1 L and 120 ml, respectively. Figure a. is reduced anti-ActRIIB and figure b. is reduced anti-Fc. Anti-Fc blot shows additional bands containing just the Fc above 25 kDA.

As stated above, based on the similarities seen in 1 L vs 120 mL control cultures and the improvement seen with 120 mL test linker cultures, the material produced from the 120 ml cultures for hIgA1 hinge and (AP)x11 and the material from 1L cultures for the control linkers were used for characterization further. A side-by-side comparison of the condition media from all four cultures was performed on SDS-PAGE and showed similar band pattern for all 4 constructs at similar intensities (Figure 11). Western blots show the protein of interest between 75-100 kDa on the non-reduced and above 50 kDa on the reduced gel as expected (Figure 12). In terms of aggregation, higher levels are seen for the test linkers after MAb purification in lane 3 (Figure 5 c, d) than controls 3 and 7 (Figure 5 a, b), which correlates with the SEC data (Figure 6). Lower molecular weight band between 25-37 kDa can be clearly seen on the blot for the test linkers in lane 3 (fig 5 c, d) but not for control linkers in lane 3 and 7 (fig 5 a, b). A comparison of

analytical SEC data after Protein A purification shows a 6-13% increase in lower molecule weight species for the test linkers (Figure 6).

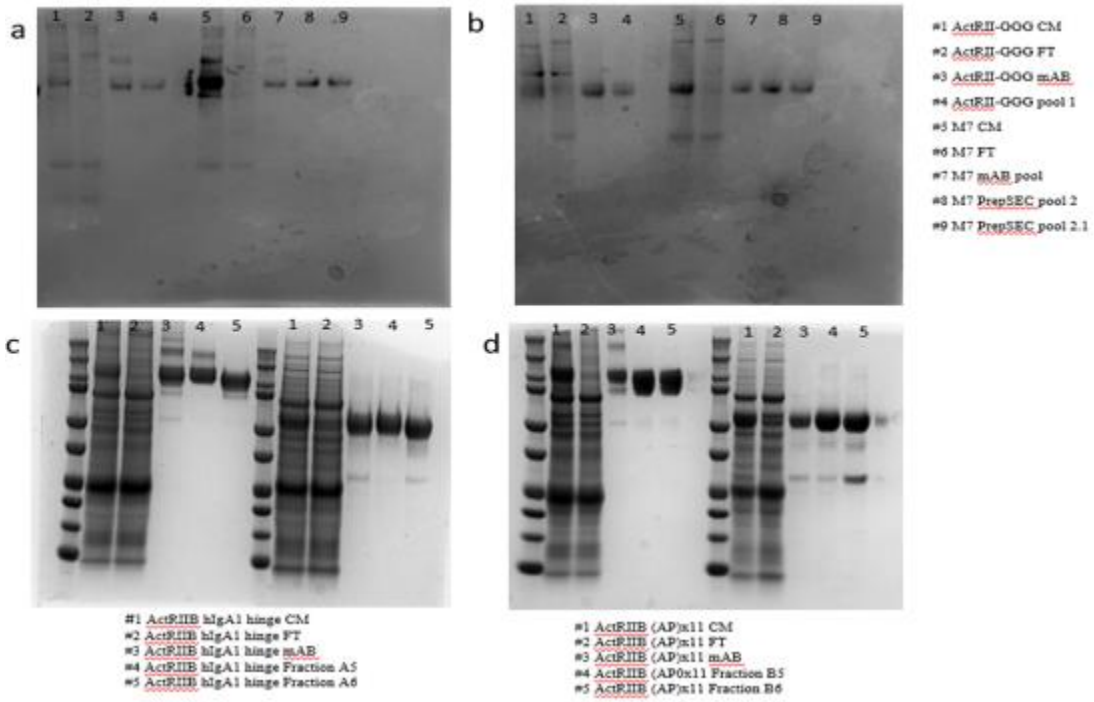

Figure 5. SDS-PAGE of 1 L Control and 120 mL Test Linker Cultures

CM, FT, MAb eluate and preparative SEC eluate for ActRIIB-GGG and ActRIIB-(G4)S4 in gels. Non-reduced controls, a. reduced controls b. ActRIIB-hIgA1 hinge non-reduced and reduced, c. ActRIIB-(AP)x11 non-reduced and reduced, d.

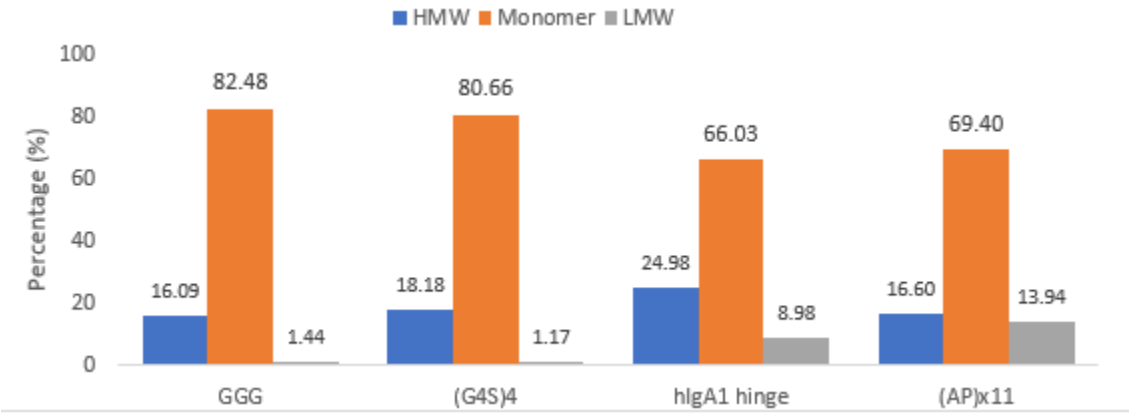

Figure 6. Quantification of Species in Culture by Analytical Sec Chromatography

Percentage of HMW, Monomer and LMW species in ActRIIB GGG, ActRIIB (G4S)4, ActRIIB hIgA1 hinge and ActRIIB (AP)x11 after MAb purification show an increase in HMW and LMW species and decrease in monomer for hIgA1 hinge and APx11 linkers

These molecules were further purified using preparative SEC to remove the aggregates and low molecular weight (LMW) species with the aim of achieving a purity of 95% monomer, an industry standard. Proteins were eluted in PBS and further characterized using SDS-PAGE gels and analytical SEC. The data shows that the control linkers are over 98% monomer (Figure 7). The main eluted peak for the test linkers were collected in two separate elution fractions and were not combined because of difference in purity. Data for the eluate that has a higher percent monomer for the test linkers is shown in Figure 7. Data for the other eluates are presented in Table 6 and 7. Analytical SEC for hIgA1 hinge shows a high molecular weight (HMW) shoulder consisting of possibly 20% of aggregates, and (AP)x11 has a lower molecular weight shoulder of about 22% consisting of possible under-glycosylated or clipped species in the eluate (Figure 15). The SDS-PAGE gels, however, show small traces of LMW species for hIgA1 hinge and some aggregation for (AP)x11. An aliquot of each eluted protein was tested on an

affinity anti-ActRIIB antibody column, which reports percentage of bound material, potentially suggesting the amount of properly folded product in each sample. Compared to GGG linker, the data shows an increase in binding of test linkers as well as (G4S)4 linker to the anti-ActRIIB affinity column suggesting that increasing length and rigidity may produce a better folded molecule (Figure 8). This material was used for binding, activity, and thermal stability assays.

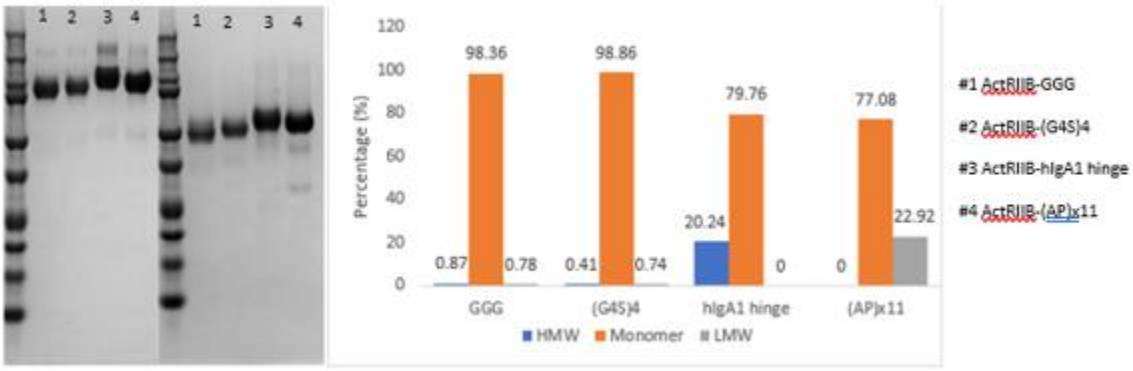

Figure 7: Characterization of Protein after Preparative Sec Chromatography

Final quantification of ActRIIB GGG, ActRIIB (G4S)4, ActRIIB hIgA1 hinge and ActRIIB (AP)x11 by SDS-PAGE and SEC showing HMW, Monomer and LMW species after preparative SEC. The control linkers have over 98 % purity and the test linkers show less than 85% purity

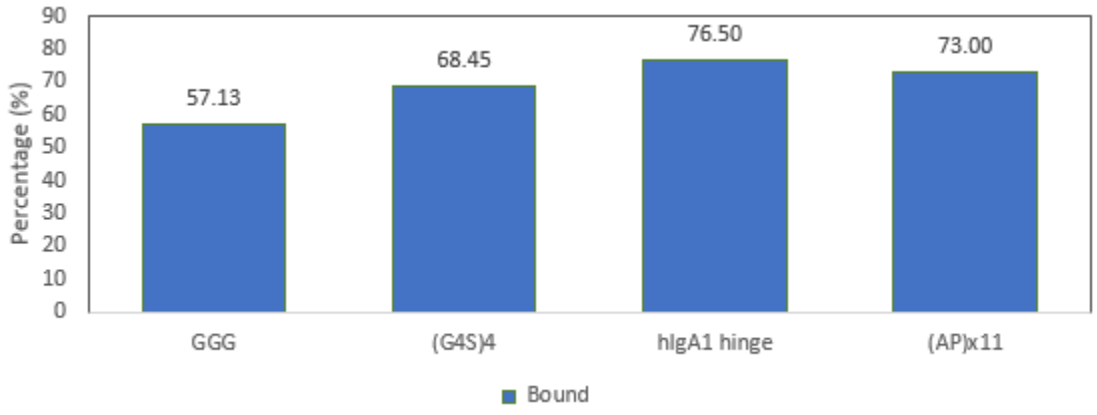

Figure 8. Results of Analytical Anti-ActRIIB Antibody Column

Percentage of bound material in ActRIIB-GGG, ActRIIB-(G4S)4, ActRIIB-hIgA1 hinge, and ActRIIB-(AP)x11. An increase in hIgA1 hinge and (AP)x11is seen compared to GGG linker

Binding and Inhibition of Ligand by ActRIIB Molecules

Surface Plasmon Resonance (SPR) was performed to assess changes in the binding induced by the incorporation of longer, rigid linkers. ActRIIB-linker molecules were tested at 10 µg/mL with a target capture RU of 70 on anti-human Fc CMS sensor chip at 37 ℃. Growth Determining Factor (GDF) 11, Activin A and Bone Morphogenetic Protein (BMP) 9 at 20nM- 0.0027 nM range of concentrations were flown over sensor bound to ActRIIB-linkers molecules. The on-rate (ka= 1/ms), off-rate (kd = 1/s) and equilibrium constant (KD) were calculated for each molecule and analyte pair based on the 1:1 fit model.

The calculated on-rates, off-rates and KDs are very similar and within two-fold instrument error for all molecules tested against GDF11 and Act A; however, GGG linker shows slightly lower affinity for BMP 9 compare to (G4S)4, hIgA1hinge and (AP)x11 (table 8). Although no significant changes to the binding of the molecules to any of its

ligands are expected, since the binding sites of these ligands were untouched, but any change made to the molecule warrants a test of its activity. Ideally, good binders have faster on-rate and slower off-rate. All the molecules had a faster on-rate and a slower off-rate indicating a good KD (Table 8). However, the sensogram for BMP 9 had slight differences (Figure 16). For instance, a slightly slower on-rate is seen for (AP)x11 compared to the control, and a slightly faster off-rate is seen with for the GGG linker compared to (G4S)4, hIgA1 hinge and (AP)x11. These differences fall within the 2-fold error range of the assay, so there is no significant change in BMP 9 binding. The caveats with BMP 9 assay are that it shows the poorest fit among the three ligands and lower Rmax than the other ligand. Overall, there is no change in binding of GDF11, Activin A and BMP 9 to ActRII-hIgA1 hinge and (AP)x11 linkers.

Activity of these molecules was measured using a luciferase reporter gene under the control of the CAGA12 promoter for GDF 11 and Activin A, and BRE promoter for BMP 9. The output is relative luciferase units (RLU), which is plotted for each concentration in the dilution curve. The linear part of the curve is used to calculate the concentration of the inhibitor where half of the maximum response (IC50) is seen for each ligand. There appears to be no differences in inhibition of GDF 11, Activin A and BMP 9 with any of the linkers. The graph for each molecule appears to be comparable (Figure 17) and the calculated IC50s are within the 2-fold error range (Table 8).

Table 8. Summary Table of Binding and Inhibition of ActRIIB Molecules

	GDF11			
Linkers	kd (1/s)	KD (M)	Rmax	IC50 (nM)
GGG	5.99 E-4	2.35 E-11	31	0.65
(G4S)4	3.61 E-4	2.02 E-11	24	0.32
hIgA1 hinge	4.77 E-4	2.67 E-11	32	0.36
(AP) x11	4.06 E-4	3.37 E-11	33	0.33
	Activin A			
	kd (1/s)	KD (M)	Rmax	IC50 (nM)
GGG	2.37 E-4	4.14 E-11	28	0.48*
(G4S)4	1.97 E-4	3.06 E-11	23	0.42
hIgA1 hinge	2.13 E-4	2.99 E-11	28	0.33
(AP) x11	2.12 E-4	3.20 E-11	30	0.31
	BMP 9			
	kd (1/s)	KD (M)	Rmax	IC50 (nM)
GGG	1.42 E-3	1.3 E-10	12	6.56
(G4S)4	8.65 E-4	8.49 E-11	11	4.56
hIgA1 hinge	8.97 E-4	4.72 E-11	12	5.82
(AP) x11	7.55 E-4	8.89 E-11	19	5.82

*Kd= off rate, KD= binding affinity, Rmax= maximum response of the analyte based on ligand level, IC50= concentration as 50% saturation of the receptor by the ligand. * Indicates the IC50s values were extrapolated.*

Thermal Stability and Hydrodynamic Diameter of the Rigid Linkers

Tm, detected by the UNCLE using the intrinsic fluorescence of aromatic amino acid, is reported to be about 69-71 °C for the control and rigid linkers (Figure 9). This data correlates well with the Tm measured by DSC, which measures heat absorption during denaturation (Table 9). The Tagg, measured by SLS, is the temperature at which 10% aggregation is detected in solution. Tagg for the control and test linkers at 85 ℃ is 71-73 ℃ range (Figure 9). Tagg for the samples tested is similar to the Tm and is reported to be about 70 ℃. Z-diameter at 25 ℃ is comparable for all samples, but there is a 6 nm increase in the rigid linker over the 4 nm increase seen for flexible linkers at 85 ℃ indicating that the rigid linkers might be expanding to a slightly bigger size suggesting

the possibility of some aggregated species (Figure 10). PDI (polydipersity index) data shows that all samples are monodispersed and majority of it is one size particles (Figure 10). Overall, all four constructs appear to be very stable at temperatures up to 85 °C with the possibility of some aggregation at higher temperatures.

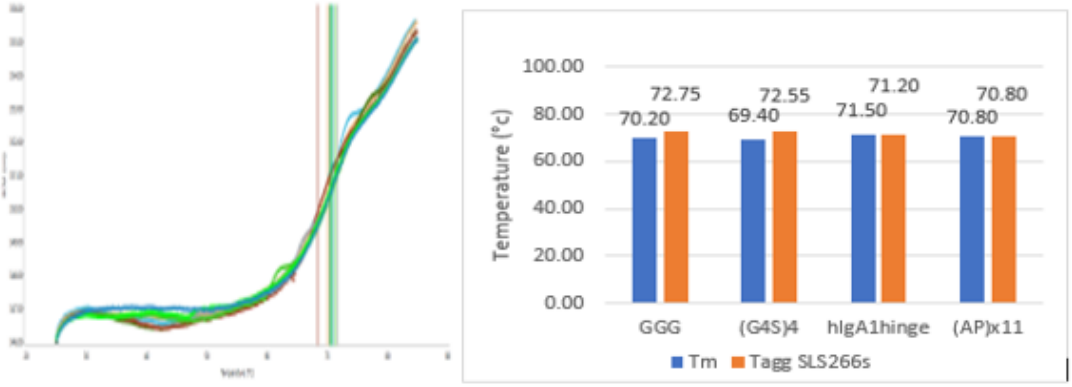

Figure 9. Thermal Stability Data from the UNCLE

Thermogram of Tm for all four constructs. Bar graph comparing Tm and Tagg for ActRIIB GGG, ActRIIB (G4S)4, ActRIIB hIgA1 hinge and ActRIIB (AP)x11 measured by intrinsic fluorescence and SLS at 266 nm wavelength.

Table 9. Tm of AcRIIB Molecules Determined by DSC.

Linkers	Tm1 (°C)	Tm2 (°C)
GGG	69.68	81.74
$(G4S)_4$	70.08	81.77
hIgA1 hinge	70.37	81.81
(AP) x11	70.04	81.68

Melting temperature of the ECD and Fc domains show comparable results for all four constructs

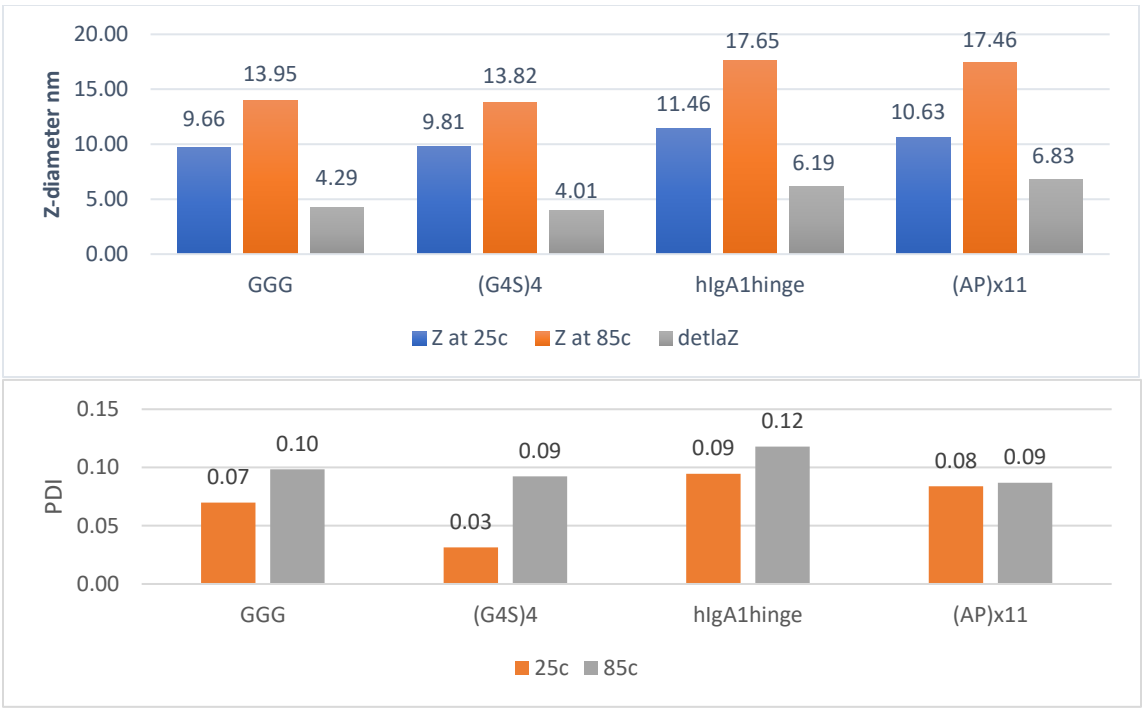

Figure 10. Measuring Z-diameter and PDI Using the UNCLE

No difference in Z-diameter is seen with the flexible vs rigid linkers at 25℃. 4nm to 6nm increase in size at 85℃, a. PDI at 85℃is slightly higher than 25℃indicating possible aggregation and fragmentation. Samples are considered monodispersed at 25℃and 85℃in b.

Result Summary

In summary, there are notable differences in cultures for the control and test linker constructs in both 1 L and 120 mL culture volumes. Cultures expressed in the 1 L volume were in large vessels and had lower yields and high percentage of LMW species for the rigid linkers compared to the flexible linkers. Among the 120 mL cultures, the (AP)x11 construct had an almost 2-fold increase in yield compared to the test linkers, while the hIgA1 hinge had comparable yield to the controls, but the LMW species are still higher than the controls. Comparing 1 L and 120 mL cultures for each, the controls appear to be

more comparable in terms of yield, aggregates, monomers and LMW species. While the test linkers had an increase in yield and slight increase in aggregation, but slight reduction in LMW molecules. The trend between the controls and test linkers continues in terms of LMW species.

After the final purification step, the control linkers were over 95% pure while the test linkers show over 79% and 77% purity for hIgA1 hinge and (AP)x11, respectively. The purity is affected by about 20% of HMW shoulder in higA1 hinge and 23% of LMW shoulder species in (AP)x11.

The changes in length and rigidity of the linkers did not change the binding and inhibition of the ligands. It also did not change the thermal stability of the molecules. An assessment by intrinsic fluorescence and by DSC shows similar Tm for all the molecules at about 70 ℃. Tagg for the molecules are also very similar. There was no significant difference in hydrodynamic radius at 25 ℃, but the increase in radius of over 50% for the rigid linkers at 85 ℃ indicates some aggregation. PDI data indicates samples are mostly monodisperse at 25° C, and also at 85 °C, suggesting majority of the sample consists of the same species in turn implying low amount of aggregation in the final product. Overall, there is improvement in binding to anti-ActRIIB affinity column with both rigid, long linkers compare to ActRIIB-GG and increase in titer with (AP)x11. However, an increase in the amount of lower molecular species was also observed and no changes were observed in terms of thermal stability.

Chapter IV.

Discussion

As observed in this study, ActRIIB-Fc with GGG linker is a comparatively well-behaved molecule with about 16% aggregation and less than 1% LMW species seen after the first step of purification, but only about 57% of this material, produced in ExpiCHO cells, bound to the anti-ActRIIB affinity column. By replacing the small, flexible GGG linker in the ActRIIB-Fc molecule with long rigid linkers, such as hIgA1 hinge and (AP)x11, the goal was to achieve a better expressing protein in ExpiCHO cultures. Improved protein expression implies high titers, but also higher percentage of correctly folded monomers and low percentage of impurities in culture. Based on the data presented in this study, increase in titer was seen for (AP)x11 at 120 ml culture volume, however this correlated with an increase in LMW species, decrease in monomer and no improvement in aggregation compared to the controls. hIgA1 hinge linker had similar titer as the control, but the remaining parameters were like that of (AP)x11. hIgA1 hinge showed slightly higher percent aggregation and low molecular weight species compare to the control. Additionally, there appears to be no improvement in titer and a decrease in the percent monomer compared to the controls. Compared to (AP)x11, hIgA1 hinge shows similar percentage of monomer, but a slight increase in aggregation and decrease of LMW species, as well as over 50% decrease in titer. Both hIgA1 hinge and (AP)x11 showed an improvement in the amount of bound material to the anti-ActRIIB affinity column, suggesting that an increase in linker rigidity promotes an increase in the expression of well-behaved product, and potentially having an impact on protein folding. It is also interesting to note that there is an 11% increase in binding to anti-ActRIIB

affinity column by (G4S)4 control in comparison to GGG control suggesting the possible influence of not just rigidity but also the length of the linker in folding of the molecule. These results were taken from 120 mL cultures, but the larger 1 L cultures were similar in terms of percent homodimer, but a decrease in aggregation and increase in percent of LMW species seen in 1 L cultures for both rigid linkers compared to 120 mL cultures, and similar binding to anti-ActRIIB affinity column was observed mostly for hIgA1 hinge, and not for the 1 L (AP)x11 culture, due to the homogeneity of the sample. Overall, the data presented in this study is suggestive of the potential effects of long, rigid linkers on the improvement in titer and folding of the molecule.

Factors Affecting Yields and Impurities during Cell Culture

Although the goal was to study the effects of protein design on protein quality, we gained insight on how culture design can also affect protein quality by evaluating the difference in two culture volumes and the factors that could influences these outcomes. The ExpiCHO protocol from ThermoFischer recommends different shake speeds (80 rpm vs 120 rpm) and vessel sizes (3 L vs 500 mL) depending on the volume of the culture. The protein quality for the control linkers remained the same, but the titer and low molecular species for the two test linkers were significantly different. The 1 L cultures had low titers from cells that remained healthy for the entire duration of the culture, and a higher percentage of LMW species was also seen, while the 120 mL culture gave higher titers from equally healthy cultures, and a 2-fold decrease in low molecular weight species for both rigid linkers. These observations warrant an evaluation of factors that could influence this outcome.

There are many internal and external factors that could affect the quality of the protein. These include the quality of the DNA, cell handling, cell health during culture and on the day of transfection, transfection efficiency, flask size and shake speed. ThermoFischer recommends that ExpiCHO cells should be handled gently rather than vigorously swirling, shaking, and pipetting. They also recommend maintaining cell viability of over 95% when carrying cells and during transfection. The optimal complexation time of 5 mins is recommended for DNA and transfection reagent, and the use of different shake speeds depending on culture volumes and orbital diameter of the shaker platform. Other external factors such as excess heat emitted by the shaker, fluctuation in incubator temperature from 37 ℃, air circulation in the incubator, gas exchange between flask and the incubator, the amount of CO2 inside the flask and in the culture, and mechanical shear generated by the movement of culture on the shaker platform (Inglebert et al., 2020).

Among the many factors listed above, the factors that may have impacted the titer of the 1 L rigid linker cultures could be the difference in heat emitted by different shakers and the inaccuracies involving old equipment. Due to large vessel size for 1 L culture, the four cultures had to be placed on different shaker platforms adding a potential source for variability, while all four 120 mL cultures were on the same shaker. There is also the possibility of inconsistent heat supply between the top and the bottom shaker platforms given the age of the incubators. These need to be confirmed with further testing.

Mechanical shear induced by difference in surface area and shaking speed of the two culture volumes could also be a factor in difference in quality. There has been abundant research done in exploring the effects of mechanical shear on protein structure

during culture. These suggest the potential of exposed residues leading to aggregation via self-interaction or fragmentation via proteolytic cleavage, if refolding is slow (Inglebert et al., 2020, Cromwell et. al., 2006) The gas-liquid interface in a flask is also critical, where any agitation at this interface can lead to cellular aggregation. (Mahler et. Al., 2005). This agitation could be from shaking of the culture, which is necessary for renewing the interface with fresh cells and for mixing in CO2 gas throughout the culture, but this type of stress could potentially expand the cells at the interface and unfold the protein that is being synthesized (Mahler et al., 2005). Large vessel size also implies increase in power of the agitators in culture, which in turn increase the mechanical stress and cell damage in larger system (Clarkson et. al., 1999). These studies all suggest that mechanical shear brought on by shaking speeds, large gas-liquid interface and foaming that occur in culture can leave certain parts of the protein exposed to form aggregates and susceptible to protease activity. These effects could be amplified by the rigidity of the test linkers themselves. The mechanical shear exerted by the movement of 1 L culture volume in a 3 L flask, creating a large gas-liquid interface, might not be ideal for exposed rigid linkers recommend shake speed by ThermoFisher might not be optimal for rigid molecules for both culture volumes, possibly exerting mechanical shear on the molecule. These theories could provide a plausible explanation for why there was no improvement in aggregation and an increase in LMW species between the control and rigid linker, but it requires additional testing.

Sequence and Length Liabilities of Long and Rigid Linkers

Inherent factors within the sequence of hIgA1 hinge and (AP)x11 linkers could potentially also affect the percentage of aggregation and LMW species seen in culture. These factors include hydrophobicity, length, amino acid composition and secondary structure of the linker, which contributes to its influence on the attached domains (Klein et. al., 2014). These could be in the form of post-translational modification within the linker region, such as glycosylation, protease cleavage sites and isomerization; all of which can affect the quality and yield of the protein.

Evaluating these inherent features of hIgA1 hinge and (AP)x11 linkers by considering some of the published research on linkers and characteristics affecting stability could assist in better understanding and designing of linkers for future studies. Human IgA1 hinge is 19 amino acids long and comprised of equal number of hydrophobic residues in the form of prolines and polar residues, which makes it hydrophobic and semi-flexible. It contains multiple glycosylation sites, which adds to its rigidity and makes the hinge more hydrophilic (Woof et al., 2011). (AP)x11, in comparison, is a rigid linker that contains 22 hydrophobic amino acid without any sites for N- or O- linked glycosylation. Both rigid linker sequences have potential sites for protease activity, which makes them susceptible to clipping in culture with a possibility that hIgA1 hinge linker is slightly protected due to glycosylation (Figure 19).

George et al., examined a set of 1280 linkers from 638 multi-domain protein and extracted common features of different linkers found in nature. They inferred and compiled a list of common characteristics and preferences based on the length of the linkers, including number of residues per size category, preference for hydrophobicity

over hydrophilicity, and percent of solvent exposure. They found small linkers consisting of 4.5+/-0.7 residues have solvent accessibility of 14.4%, while medium linker of 9.1+/-2.4 residues have solvent accessibility of 26.3% and large linkers of 21.0 +/- 7.6 residues with a solvent accessible of 42.5% with an average linker length noted in the dataset was 10.0 +/- 5.8 residues (George and Herringa et al., 2002). Typically, small linkers tend to be more hydrophobic, such as the GGG linker in this study comprised of glycine residues, while long linkers tend to be more hydrophilic. There is a preference for Cys, Asn, Gln in the linker region, and a reduced preference for hydrophobic amino acids depending on the length (George and Herringa et al., 2002). Most linkers in nature tend to be partially buried with a solvent accessibility of 26.7% vs 45.6 % depending on adjacent domains separation. Small linkers affect the folding and unfolding kinetics, with increased rates seen with shorter linkers (George and Herringa et al., 2002)

In examining the influence of the length, 3 vs 19-22 residues, and the amino acid composition of the linkers on the stability of the molecule, A study conducted by Robinson et al. showed both were factors influencing stability of the linker and the domains attached. They reported that shortened linkers, from 59 residues to 13 residues, increased the rate of folding detected by urea denaturing by circular dichroism (CD). This is because shorter linker allows for a smaller conformation space therefore limiting the number of different conformations which can be assumed by unfolding protein before they refold, thus increasing the rate of folding. However, a linker that is too short may have added strain in maintaining the native structure (Robinson et al., 1998). The results of this study suggest that it is crucial to design a linker with appropriate length needed for stability and enhanced folding of the molecule.

The observations made in the two studies mentioned above allows us to theorize that a combination of these factors could correlated with observed increase in LMW species and lack of improvement in aggregation seen in this study with rigid linkers. The potentially slower folding rate of long linkers and the length, rigidity, and hydrophobicity content can expose the linker to solvents containing proteases, which are enzymes that cleave and fragment proteins. While hIgA1 has the advantage of glycosylation as a measure to protect against proteolytic cleavage, (AP)x11 is void of such an advantage and this can be modestly supported by differences in LMW species observed for hIgA1 hinge, which was consistently lower than those seen for (AP)x11 in both 1L and 120 mL cultures. Based on the research presented here, an ideal linker will be, on average, 10-15 amino acids long, containing residues that makes the linker more hydrophilic in nature, and would be void of any potential protease sites.

Effect of Glycosylation Sites and Xaa-Pro Pairing on Folding

Contrary to expectation, no improvement in aggregation was seen with hIgA1 hinge or (AP)x11 linkers compared to the controls, although there was an increase in binding anti-ActRIIB affinity column suggesting an increase in correctly folded ActRIIB molecule. The two sequence features of hIgA1 hinge and (AP)x11 that might have a potential role in this outcome are the glycosylation of serine and threonine residues, and the paring of proline with its adjacent amino acids in the peptide. Both factors play a critical role in slowing down isomerization and protein folding, which could potentially explain why there was no improvement in aggregation with rigid linkers.

Increase in glycosylation leads to increase in aggregation as seen in multiple studies (Spahr et. al. 2014, Spencer et. al., 2013, Tyshchuck et. al., 2017). The sequence of hIgA1 hinge is semi-flexible that contains serine and threonine residues, which are potential sites for O-glycosylation. Glycosylation, however, is required for proper folding and functioning of a protein, and it also serves an important role in making unique structural modification to the protein backbone by adding monosaccharide, disaccharide and oligosaccharide (Johnson et al., 2014). Glycosylation patterns and the extent of glycosylation can vary with each culture and affect the level of aggregation. A study conducted by Johnson et al., shows the complexity of carbohydrates added to serine residues of hIgA1 hinge can affect the structure, conformation, and isomerization of the peptide in-turn affecting the rate of folding and aggregation. By measuring end-to-end distant distribution (dÅ), radius of gyration (r(Å)), arm angle (an angle between the two arms of the molecule), and dihedral angle, they observed addition of simple sugars, such as GalNac, on all five serine residues in hIgA1 hinge linker showed the most extended conformation with a dÅ of 20-70 Å and the biggest size as measured by r(Å) in comparison to unglycosylated and disialyated hIgA1 hinge linker. The arm angle of greater than 90 degree gives the molecule a stiffer, elongated I-shape confirmation. This compares to hIgA1 hinge that is heavily glycosylated with disiaylated core, containing GalNac, Gal and NeuAc sugars, giving it a more rigid conformation with d(Å) of 20-55 Å and has a more restricted conformational arm angle of 15-120 degrees, where the two domains are perpendicular to each other to avoid steric clashes between the sugar rings. Adding simple or complex O-glycan on semiflexible linker makes it rigid by restricting it conformational movement and ensuring separation of the attached domains by either

taking on a perpendicular or elongated conformation compared to the unglycosylated hIgA1 hinge. However, heavily glycosylated serine residues in hIgA1 hinge have slower cis-trans isomerization, which affects the folding of the molecule leading to aggregation. In addition, the stiffness and extended confirmation taken on by glycosylated hIgA hinge linker exposes it to the aqueous environment and to proteolytic cleavage.

In addition to the finding of Johnson et.al., which is heavily focused on O-glycosylation of the serine residues, the threonine residues have equal potential to be O-glycosylated and alter the conformation of the proline at the C-terminus to a trans conformation. A study reports 2 or more N-linked and 3-5 O-linked glycan are found on hIgA1 hinge with most common sites are as follows: T225, T228, S230, S232 and T236 (Novak et al., 2000). The most common glycan moieties attached are Gal-GalNac disaccharide, mono- and disiaylated core (Novak et al., 2000). In fact, it has been reported that attachment of GalNac to T228 and T236 in the hinge region of hIgA1 (PSTPPTPSPSTPPTPSPS) makes it more hydrophilic and better suited for aqueous environment (Ohyama et al., 2020) suggesting that, in addition to glycosylated serine residues, adding GalNac to specific threonine residues allows the linker to be more hydrophilic and slightly more suitable for aqueous environment. These studies taken together implies that hIgA1 linker benefits as well as suffers from having up to 5 glycosylation sites depending on the number of glycosylated site and moieties added to each site. These modifications on serine and threonine residues can lead to a more elongated and rigid structure needed for domain separation and allow the linker to be more hydrophilic making it suitable for aqueous environment, while negatively impacting it by exposing it to proteases and lowering rate of folding.

Peptidyl-prolyl bond, a peptide bond between proline and the residue preceding it, is also critical for folding rates and cis-trans isomerization of this bond and can present as the rate-limiting step in protein folding. The cyclic side chain of the proline residue imposes confirmational restriction making it incapable of contributing to the secondary structure in a manner like the other amino acids via H-bond formation (Wriggers et al., 2005, Radford et al., 1987). This feature makes proline the most favored amino acid in linker region for purpose of adding rigidity, but an excess number of proline residues might be problematic due to possible intermolecular interaction between the available H-bond within adjacent domains (Robinson et al. 1998). Each amino acid in the peptide can exist in cis or trans confirmation with a preference for trans confirmation by all, except proline. It is energetically favorable for proline to exists in the cis conformation (Lhoste et al., 2021). Several studies, including the study conducted by Reimer et.al, examined the influence on the cis prolyl bonds of amino acids preceding proline (Xaa-Pro) as well as the influence of all the local amino acids in the peptide that are in cis conformation and the influence of distant amino acids on cis propyl bond during a folding event. This study also provides an insight on the type of amino acids that would accelerate and deaccelerate the cis propyl bond involving proline. For instance, an Ala-Pro bond is a 3-fold weaker catalysis than Pro-Ala in a A-P-P motif; while Pro-Pro bonds are comparatively accelerated if preceded by alanine in A-P-P motif. Pro-Pro in cis conformation can accelerate the rate of conversion of Ala-Pro from cis to trans; while the reverse, where Ala-Pro are in cis conformation, there is a 1.5x decrease in rate of isomerization of the Pro-Pro bond following the Ala-Pro bond (Reimer et al., 1998) This data indicates that

Ala-Pro bond could potentially be the rate limiting step in folding of (AP)x11 linker exposing the linker to protease activity.

The same group looked at the contribution of other amino acid in the Xaa-Pro position and reported that accelerated isomerization can be achieved by pairing proline with amino acids that are electron donating, such as Asp and Glu; amino acid with small side chain, such Ala and Gly, and other amino acids, such as Asp, Ser, Thr, Asn, Cys have also shown an increase in isomerization with Ala being the least preferred residue in the linker region of naturally occurring linkers (Reimer et.al., 1998, George and Herringa et al., 2002). Cys are problematic, in general, due to its involvement in disulfide bond and structural complications from disulfide scrambling. Another study reported an increase in isomerization catalysis if the preceding amino acid is hydrophobic, rather than negative charged or polar residues, as hydrophobic are also reported to be preferred residues in linker region of naturally occurring linkers (Jakob et al., 2009, George and Herringa et al., 2002). There is a preference for some of these pairing more than others depending on the overall secondary structure of the linker, such as Pro-Pro, Trp-Trp, Met-His, Gln-Pro, Pro-leu are preferred in non-helical linkers; while Asp-Pro, Glu-Pro are preferred in helical linkers (George and Herringa et al., 2002). These results taken together suggest that Ala-Pro paring in (AP)x11 might be inducing aggregation by slowing down isomerization rate.

Analyzing linker sequences in accordance with the results of these studies mentioned above, hIgA1 hinge has TPPTPT motif which may have a comparatively better isomerization rate than Ala-Pro pairing in (AP)x11; Threonine is highly preferred in linker region over Alanine and has a higher isomerization rate, which will influence

the rate of isomerization of the Pro-Pro bond in a T-P-P motif (Johnson et al., 2014, Argos et al., 1990). When glycosylated, threonine in hIgA1 hinge can alter the confirmation of the proline residues in its C-terminal to trans; while proline can exist in both conformations, but it prefers cis confirmation (Ohyama et al., 2020). The energy required for this confirmational change could have a negative impact on the rate of folding. However, the glycosylation in hIgA1 hinge is thought to have protective benefits to the linker from protease activity and increase the hydrophilicity of the linker (Woof et al., 2011). These observations could potentially explain the results of this study that show a trend towards a decrease in LMW species seen in culture for hIgA1 hinge compared to (AP)x11. The data taken together suggests that a combination of factors, such as Thr-Pro/Ser-Pro pairing as well as glycosylation sites among other factors, may provide increase protection to the hIgA1 hinge leading to lower percentage of LMW species compare to (AP)x11. (AP)x11, on the other hand, is a long, rigid, hydrophobic linker; but the length, absence of glycosylation sites within the linker and the effect of Ala-Pro pairing on folding makes it more susceptible to protease activity.

In this study, we were able to show significant improvement in the titer and binding of ActRIIB to the epitope of anti-ActRIIB antibody by replacing short, flexible GGG linker with rigid linker of 19-22 amino acids. Examination of the expression showed no major changes in aggregation, but a decrease of monomer and increase of LMW species for the rigid linkers compare to the control linkers. By continuing to optimize hIgA1 hinge and (AP)x11, we can ameliorate the level of clipping and aggregation seen in this study. Several design and culture strategies discussed in this paper can be applied to the hIgA1 hinge and (AP)x11 linkers to improve the design as

well as minimize clipping that may occurs due to prolonged exposure of elongated, rigid linker to the aqueous environment; potentially improve folding rate and level of aggregation by altering the sequences with more favorable Xaa-Pro pairing. In designing improved rigid linkers for future studies, the linker would ideally be 10-15 residues long to lower solvent exposure, with a special focus on avoiding excessive Ser or Thr residue and optimal Xaa-Pro pairing. The recommendation for the amino acid in Xaa-Pro pairing would mostly likely be the following: Asn-Pro, Gln-Pro, Asp-Pro, Met-Pro, Glu-Pro, in a mostly polar residues backbone to make the linker hydrophilic. Any changes made to these linkers based on the results of this study would mostly likely lead to further improvements in the expression of ActRIIB. Overall, hIgA1 hinge and (AP)x11 are good choices for linkers and were successfully implemented in other research molecules.

Appendix 1.

Additional Tables

Table 10: Summary of Culture Parameter from 1 L Cultures

Molecules	GGG	(G4S)4	hIgA1hinge	(AP) x11
Culture volume	1 L	1 L	1 L	1 L
Cell Count (cells/mL) day5	10.2	10.42	10.6	10.52
Viability (%) day 5	99.4	99.2	99.7	99.6
Cell Count (cells/mL) day8	8.0	8.04	8.84	9.18
Viability (%) day8	98.2	98.2	95.7	97.5

Cell count and viability reading on day 5 and day 8 for each culture

Table 11: Summary of Culture Parameter from 120 mL Cultures

Molecules	GGG	$(G4S)_4$	hIgA1hinge	(AP) x11
Culture volume	120 mL	120 mL	120 mL	120 mL
Cell Count (cells/mL) day5	11.45	10.48	11.36	11.94
Viability (%) day 5	96.3	96.6	95.7	98.5
Cell Count (cells/mL) day8	8.38	8.21	10.07	9.28
Viability (%) day8	93.7	94.6	96.1	95.2

Cell count and viability reading on day 5 and day 8 for each culture

Appendix 2.

Additional Figures

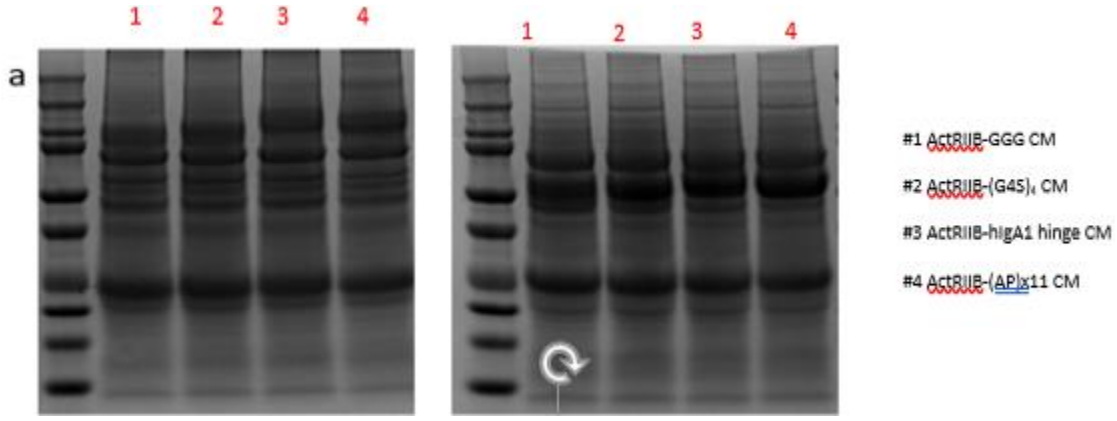

Figure 11: Analysis of the Protein in CM by Western Blot

CM of ActRIIB GGG, ActRIIB (G4S)4, ActRIIB hIgA1 hinge and ActRIIB (AP)x11 showing similar bands patterns in all four lanes

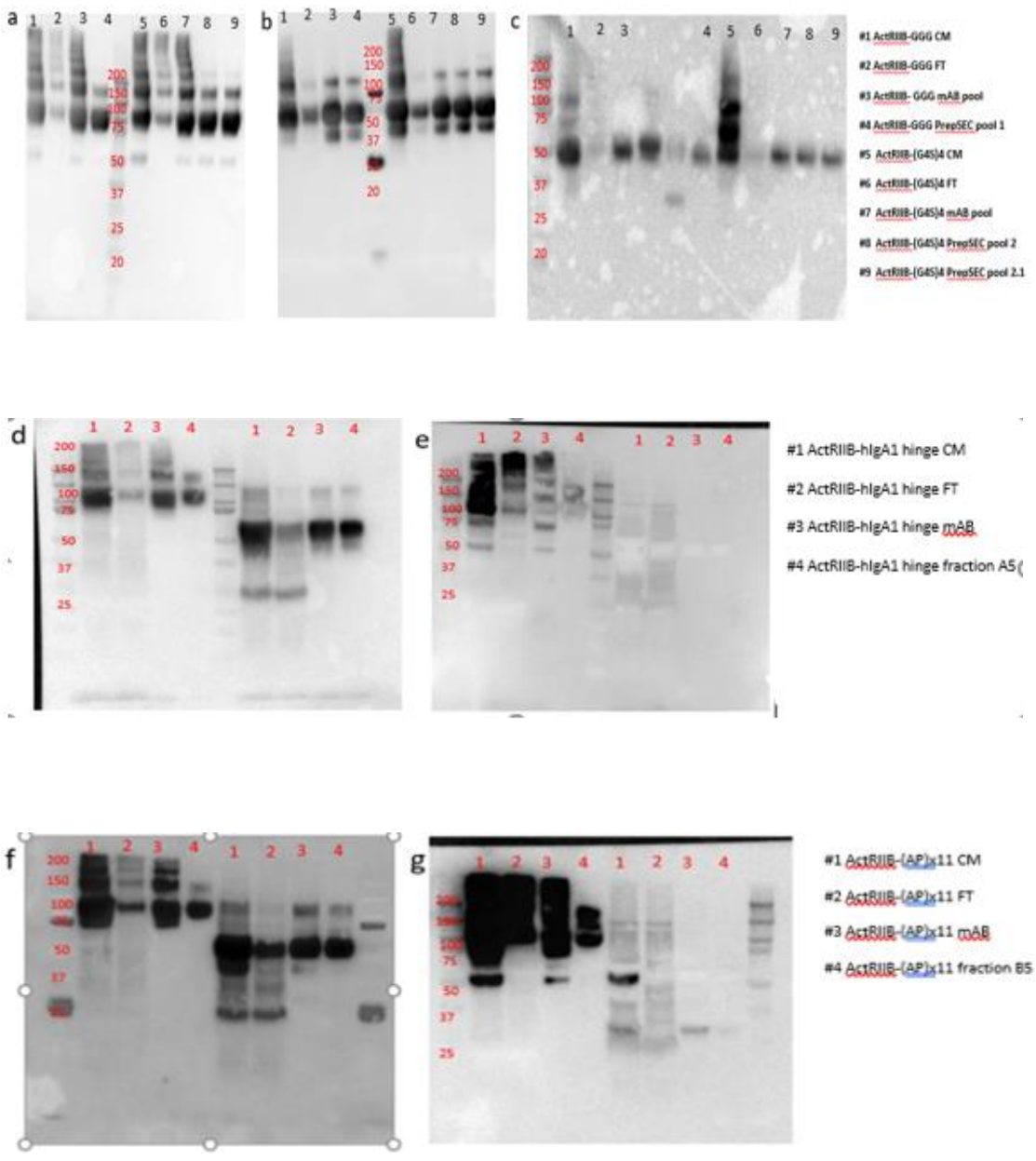

Figure 12: Analysis of CM, FT, Elution after MAb and Preparative SEC

CM, FT, MAb elute and PrepSEC elute for ActRIIB-GGG, ActRIIB-(G4S)4 blots. Anti-ActRIIB antibody non-reduced, a. reduced, b. Anti-human Fc, c. ActRIIB-hIgA1 hinge blot. Anti-ActRIIB antibody non-reduced and reduced, d. Anti-human Fc non-reduced and reduced, e. ActRIIB-(AP)x11 blot. Anti-ActRIIB antibody non-reduced and reduced, f. Anti-human Fc non-reduced and reduced, g.

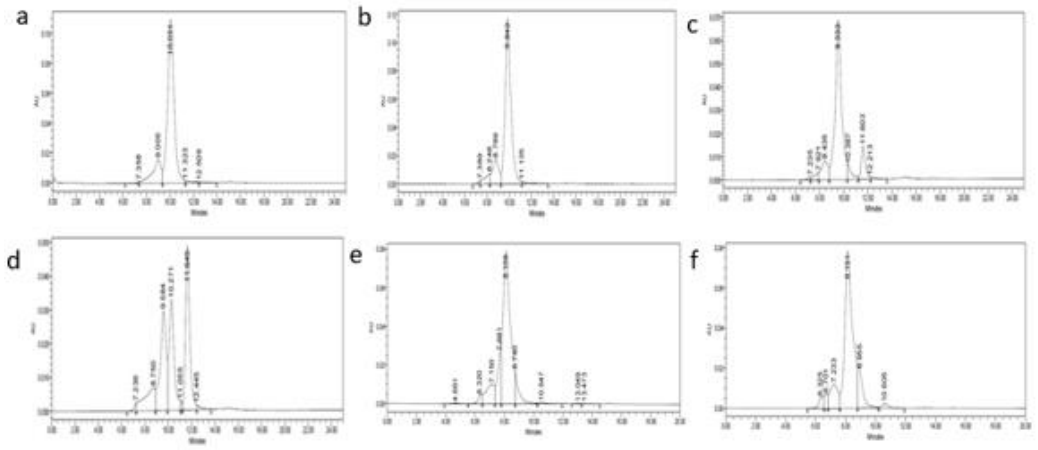

Figure 13: Analysis of Aggregation and Low Molecular Weight Species after MAb

Analytical SEC after MAb pH 3.5 ActRIIB GGG, a. ActRIIB (G4S)4, b. ActRIIB hIgA1 hinge from round 1, c. ActRIIB (AP)x11 from round 1, d. ActRIIB hIgA1 hinge from round 2, e. ActRIIB (AP)x11 from round 2, g.

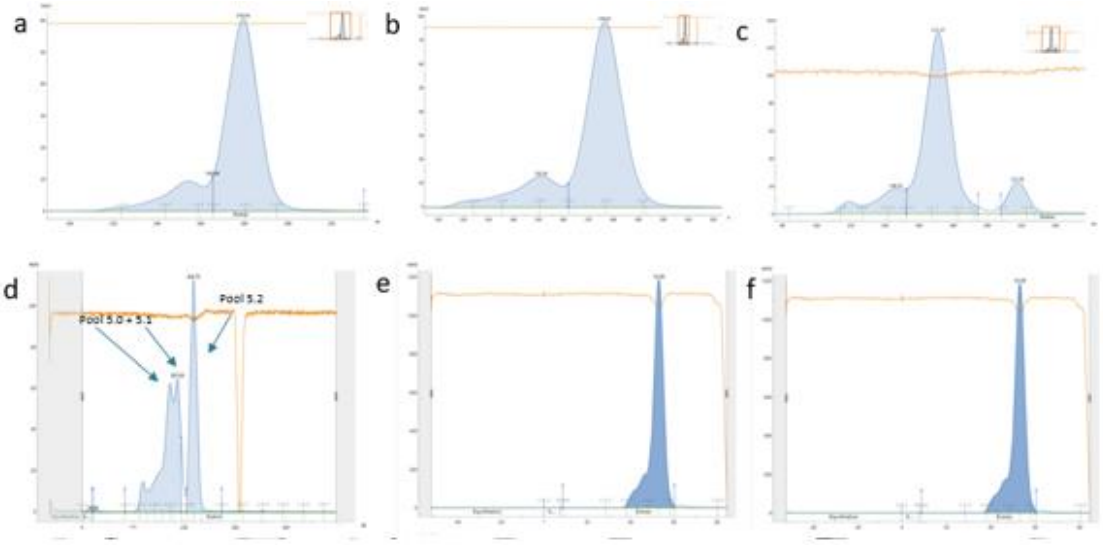

Figure 14: Chromatogram of Samples from Preparative SEC

Preparative SEC chromatogram ActRIIB GGG, a. ActRIIB (G4S)4, b. ActRIIB hIgA1 hinge from round 1, c. ActRIIB (AP)x11 from round 1, d. ActRIIB hIgA1 hinge from round 2, e. ActRIIB (AP)x11 from round 2, g.

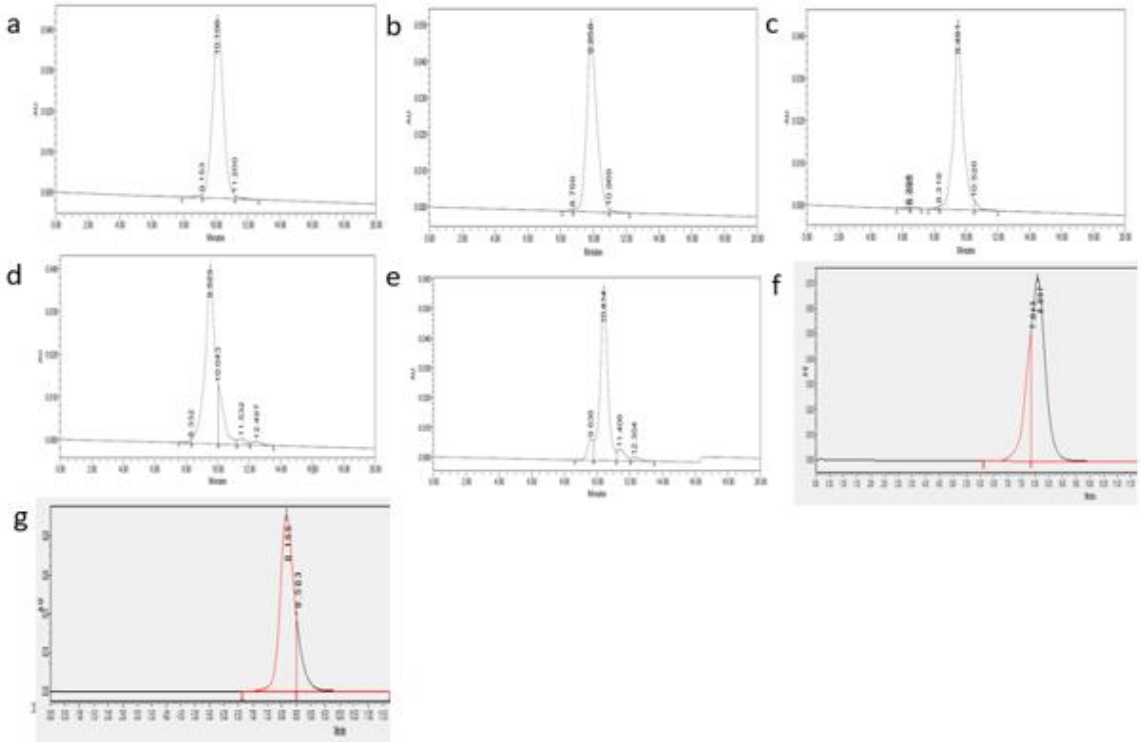

Figure 15: Analysis of Aggregation and Low Molecular Weight Species post Preparative SEC

Analytical SEC after Preparative SEC ActRIIB GGG pool 1, a. ActRIIB (G4S)4 pool 2, b. ActRIIB hIgA1 hinge pool 3 from round 1, c. ActRIIB (AP)x11 pool 5 from round 1, d. ActRIIB (AP)x11 pool 5.1 from round 1, e. ActRIIB hIgA1 hinge fraction A5 from round 2, f. ActRIIB (AP)x11 fraction B5 from round 2, g.

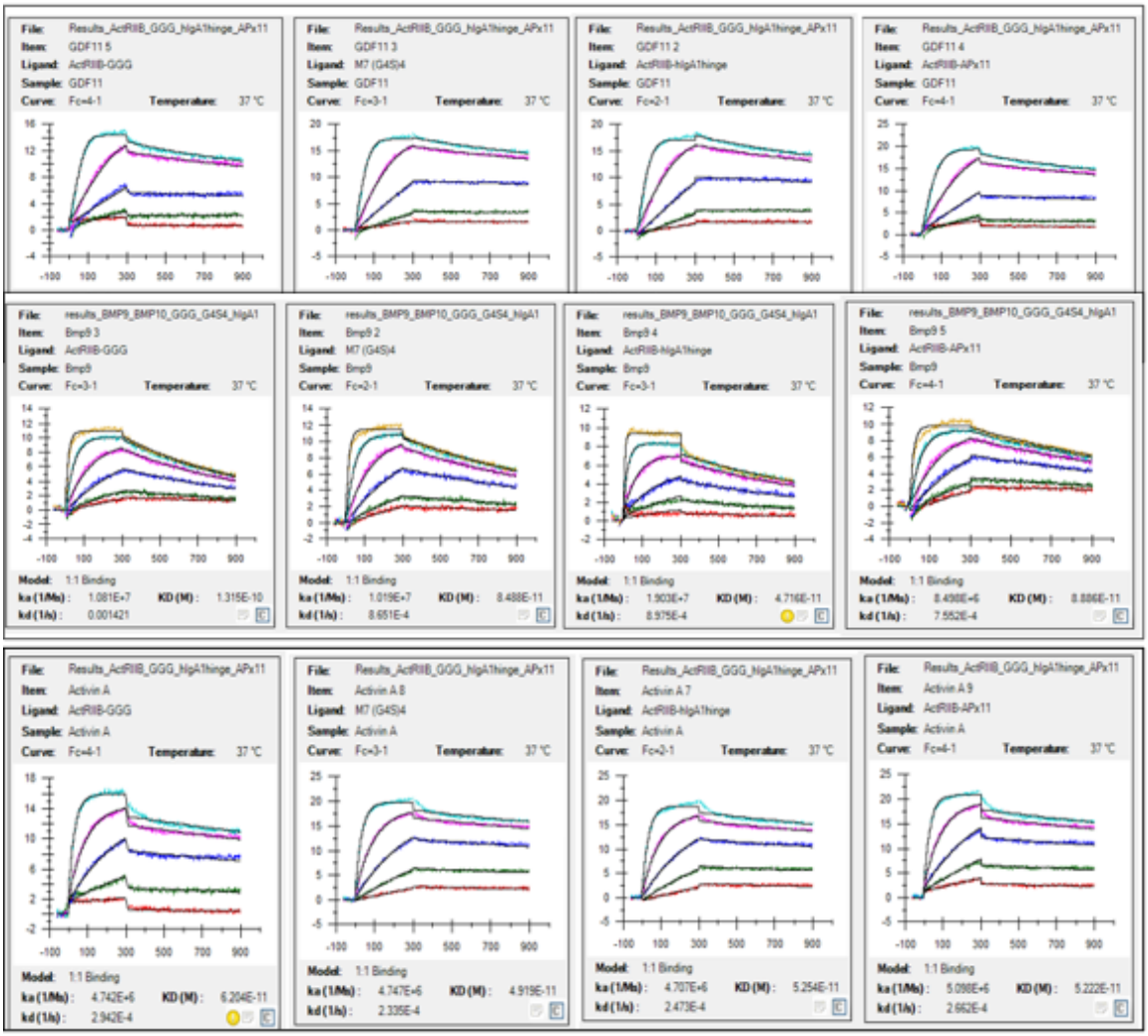

Figure 16: Binding data from Biacore

Binding of ActRIIB GGG, ActRIIB (G4S)4, ActRIIB hIgA1 hinge, ActRIIB (AP)x11 to GDF 11, BMP 9 and Activin A.

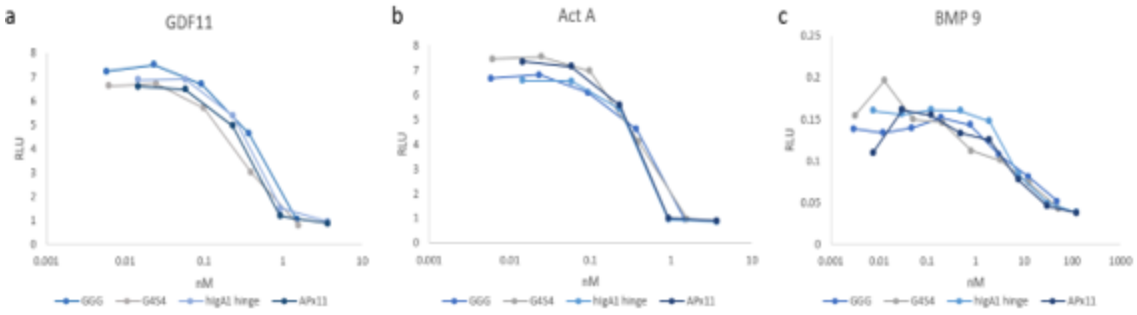

Figure 17: Inhibition Data from RGA

Inhibition of ActRIIB GGG, ActRIIB (G4S)4, ActRIIB hIgA1 hinge and ActRIIB (AP)x11 to GDF11, a. Activin A, b. BMP 9, c.

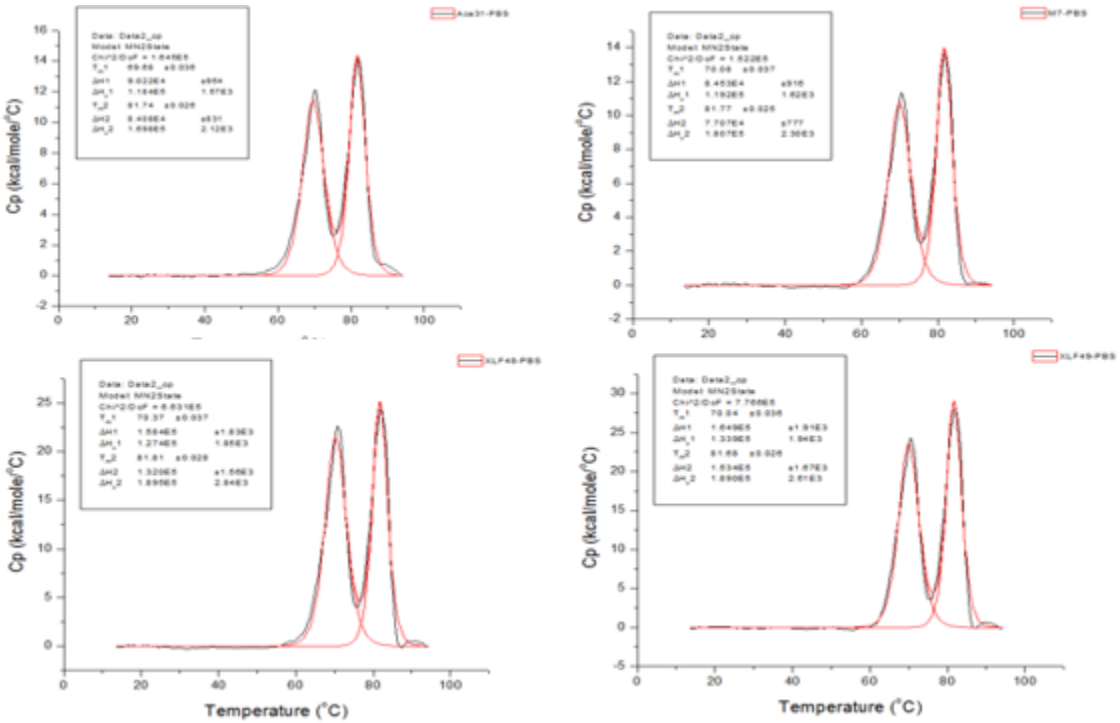

Figure 18: Thermograms from DSC.

Melting temperature (Tm) of the ECD and Fc domains of ActRIIB GGG, ActRIIB (G4S)4, ActRIIB hIgA1 hinge, ActRIIB (AP)x11. X-axis represent the change in temperature (°C). Y-axis represent the deltaH required for the domain to unfold

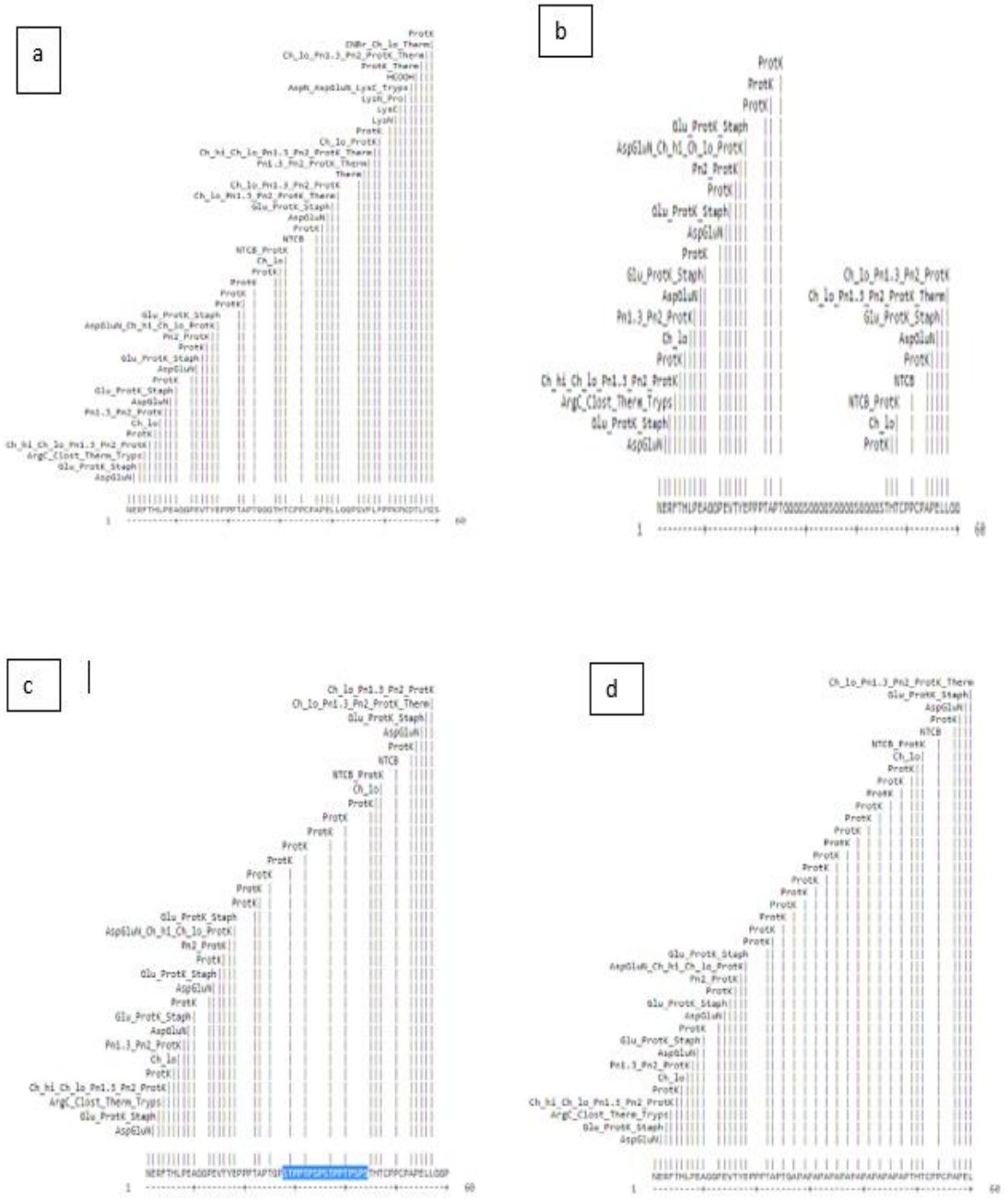

Figure 19: Proteolytic Cleavage Site Predicted by ExPasy.

Illustrates potential proteolytic cleavage sites on ActRIIB-GGG, a. ActRIIB-(G4S)4, b. ActRIIB-hIga1hinge, c. ActRIIB-(AP)x11, d.

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