Polycationic Resurfaced Nanobodies: A Potentially General Scaffold for Intracellularly Targeted Protein Discovery

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Table of Contents

Materials and Instrumentation
Materials…………………………..………………………………………………………………………S3
Instrumentation…………………………………………………………………………………… S3

Experimental Data
Cloning……………………………………………………………………………………………..S4
Protein purification……………………………………………………………………………..S4
Circular dichroism……………………..……………...…………………………………………S4
Mammalian cell culture………………………………..…………………………………….....S4
Live cell fluorescence microscopy……………………………………………………………S4
Flow cytometry analysis..S5
Cytosolic protein extraction and whole cell lysate preparation for western blot……...……....S5
Lysate Ni-NTA pull-down assay………………………………..……..………………………S5
Figure S1: Sequence information for all proteins used in this work.......................................S6
Figure S2: SDS-PAGE of Purified Proteins..S7
Figure S3: Brightfield images of mammalian cells..S8
Figure S4: Fluorescent microscopy images for supercharged GFP.......................................S9
Figure S5: ITC Data..S10

Materials and Methods

Materials
All chemicals obtained from Sigma-Aldrich unless specified
LB Miller Broth – Fisher
Phosphate buffered saline (PBS)- Corning Cell Grow
0.25% Trypsin- Hyclone/Thermo Scientific
Fetal bovine serum (FBS)- Atlanta Biologicals
Dulbecco's modified Eagle medium (DMEM)- Hyclone/Thermo Scientific
Mammalian cell culture dishes- Fisher Scientific
RIPA Buffer- Boston BioProducts
5-alpha chemical competent *E. coli*- NEB
BL21 (DE3) chemically competent *E. coli*- NEB
Agar- GoldBio Technology
Carbenicillin – GoldBio Technology
Restriction Enzymes- NEB
Isopropyl-β-D-1-thiogalactopyranoside (IPTG)- GoldBio Technology
cOmplete ULTRA Tablets, Mini, EDTA-free- Roche
Quick Ligation Kit- NEB
Vent Polymerase- NEB
Oligonucleotides- IDT
Miniprep Kits- OMEGA
All antibodies obtained from Abcam unless specified
Rab5 antibody – Cell Signaling Technologies
iBlot gel transfer stack kit, Novex
PageRuler Prestained Protein Ladder- Thermo Scientific
10% Ready Gel Precast Gels – BioRad
3T3 cells – ATCC

All water was obtained from a Milli-Q water purification system.

Instrumentation
MoFlo Flow Cytometer and High Speed Cell Sorter with a solid state iCyt 488nm laser.
Sonifer W-350 cell disruptor – Branson
Fluoresence microscopy images were taken with EVOS FL from Advanced Microscopy Group.
MJ mini gradient thermal cycler, BioRad
Molecular imager gel doc XR+ system, BioRad
Circular dichroism spectrometer, Aviv model 202
iBlot Apparatus, Invitrogen
Odyssey Classic Infrared Imager, LI-COR
iTC200, Microcal (Malvern)

Experimental Data

Experimental Procedures

Cloning

All plasmids were constructed on a pETDuet-1 backbone. All proteins were assembled from a set of overlapping oligonucleotides. Proteins were amplified using vent and the constructs were ligated into *NcoI* and *NotI* restriction enzyme cleavage sites in the pETDuet-1 plasmid. Proteins containing GFP fusions were assembled from a set of overlapping oligonucleotides and ligated into *NcoI* and *KpnI* restriction enzyme cleavage sites in the pETDuet-1 plasmid.

Protein purification

Plasmids were transformed into BL21s (DE3). Cells were grown in either 2500 or 500 mL LB cultures containing carbenicillin at 37 °C to OD_{600}= ~0.6 and induced with 1 mM IPTG at 25 °C overnight. Cells were then collected by centrifugation and resuspended in either phosphate buffer with 150 mM NaCl for NBs (20 mM Sodium Phosphate, pH 7.4) or resuspended in phosphate buffer with 2 M NaCl for pcNBs (20 mM Sodium Phosphate, pH 7.4) and stored at -20 °C. Frozen pellets were thawed and incubated with cOmplete ULTRA protease inhibitors tablets then sonicated for 2 minutes. The lysate was cleared by centrifugation (9000 rpm, 20 minutes) and the supernatant was mixed with 1 mL Ni-NTA agarose resin for 30 minutes. The resin was collected by centrifugation (4950 rpm, 10 minutes). The resin was washed with 50 mL buffer and 20 mM imidazole then 10 mL buffer and 50 mM imidazole. The protein was then eluted with 7 mL buffer containing 300 mM imidazole. The proteins were dialyzed against buffer and analyzed for purity by SDS-PAGE. Purified proteins were quantified using absorbance at 280 nm.

Circular dichosim

Proteins were purified as described above. Separately, each protein was diluted to 6-8 uM in Sodium Phosphate buffer (20 mM Sodium Phosphate, pH 7.4 and150 mM NaCl). Wavelength data are the average of three scans from 250 nm to 200 nm in 1 nm steps at 25 °C.

Mammalian cell culture

NIH/3T3 cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% Fetal Bovine Serum (FBS). All cells were incubated at 37 °C with 5% CO_2 environment.

Live cell fluorescence microscopy

Mammalian cells were grown to ~80% confluency in a 6-well plate. Cells were then washed once with PBS and 2 mL of 250 nM protein fused with GFP was added. The cells were incubated with the protein solution for 3 hours at 37 °C, 5% CO_2 environment. After the incubation period, cells were washed once with PBS and three times with PBS-HS (heparin

sulfate 20 U/mL) for 10 minutes at 37 °C, 5% CO_2. The cells were then imaged on the EVOS FL fluorescence microscope.

Flow cytometry analysis

Mammalian cells were grown to 80% confluency in a 6-well plate. Cells were then washed once with PBS and 2 mL of 10 nM, 250 nM, or 500 nM protein fused with GFP was added. The cells were incubated with the protein solution for 3 hours at 37 °C, 5% CO_2 environment. After the incubation period, cells were washed once with PBS and three times with PBS-HS (heparin sulfate 20 U/mL) for 10 minutes at 37 °C, 5% CO_2. The cells were then removed from dish with 0.25% trypsin-EDTA and collected by centrifugation. The cells were then suspended in PBS and taken for flow cytometry analysis.

Cytosolic protein extraction and whole cell lysate preparation for Western blot

3T3 cells were plated in 6-well plate and grown to ~80% confluency. The cells were treated with 250 nM or 500 nM proteins (wtNB-GFP and pcNB-GFP or wtNB and pcNB, respectively) for 24 hours at 37 °C, 5% CO_2. After treatment, cells were washed once with PBS and once with PBS-HS (heparin sulfate 20 U/mL) for 10 minutes at 37 °C, 5% CO_2 then lifted with 0.25% trypsin-EDTA and pelleted. For cytosolic protein extraction, cell pellets were resuspended in 100 µL of 50 µg mL^{-1} digitonin in 75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose supplemented with Roche protease inhibitor cocktail for 10 minutes on ice. Cells were then centrifuged for 5 minutes at 13,000 rpm. Supernatant was then used as cytosolic protein extraction. Left over pellets were then resuspended in 100 µL RIPA buffer supplemented with Roche protease inhibitor cocktail and incubated on ice for 5 minutes then further lysed through a 20 gauge needle. Supernatant was then used as whole cell lysate extraction. Both supernatants were collected and separated by SDS-PAGE and transferred to a nitrocellulose membrane via an iBlot western blotting apparatus. The membrane was incubated in 1X TBS with 5% milk at 25 °C for 1 hour. The membrane was then washed 3 times with 1X TBS and 0.1% Tween-20. Primary antibodies for GFP, Erk1/2, and Rab5 were incubated with the membrane containing GFP fused nanobodies overnight in 10 mL of 1X TBS, 5% BSA, and 0.1% Tween-20 at 4 °C. The western blot containing unfused nanobodies were incubated with primary antibodies for His6X, Erk1/2, and Rab5 overnight in same mixture. Both membranes were washed 3X with 1X TBS containing 0.1% Tween-20 and then incubated in Anti-Rabbit (Alexa Fluor 790) in 10 mL TBS, 5% milk and 0.1% Tween-20 for 1 hour at 25 °C. The membrane was washed 3X with 1X TBS containing 0.1% Tween-20 and imaged in 1X TBS using the Odyssey Classic Infrared Imager.

Lysate Ni-NTA pull-down assay

wtNB1 and pcNB1 (nanobodies for GFP) tagged with His_{6x} were cloned into MCS1 of pETDuet-1 using restriction enzymes *NcoI* and *NotI*. Untagged GFP was cloned into MCS2 of pETDuet-1 using restriction enzymes *NdeI* and *KpnI*. Completed constructs were transformed into BL21s (DE3). Cells containing the co-expressed pair were inoculated and induced as described previously. Cells were pelleted and purified as described previously. The pull-down was analyzed by SDS-PAGE.

Figure S1. Sequence information for all proteins used in this work.

NB1 (GFP NB)
MGMQVQLVESGGALVQPGGSLRLSCAASGFPVNRYSMRWYRQAPGKEREWVAGMSSAGD
RSSYEDSVKGRFTISRDDARNTVYLQMNSLKPEDTAVYYCNVNVGFEYWGQGTQVTVSSHHH
HHH

pcNB1 (GFP NB)
MQVQLVEKGGKRVQPGGSLRLKCAASGFPVNRYSMRWYRQAPGKEREWVAGMSSAGDRS
SYEDSVKGRFKIKRDDARNTVYLRMRKLKPEDTAVYYCNVNVGFEYWGQGTRVTVSKKHHHH
HH

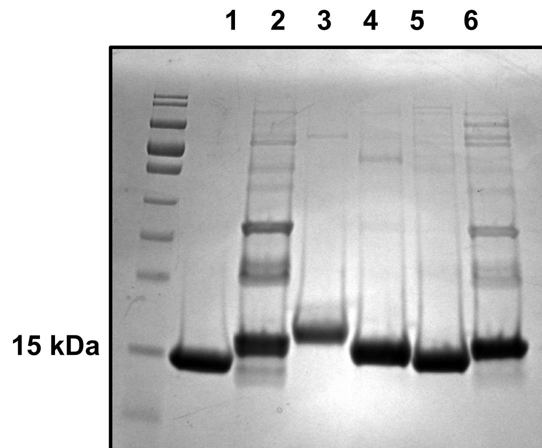
NB2 (HER2 NB)
MEVQLVESGGGLVQAGGSLRLSCAASGITFSINTMGWYRQAPGKQRELVALISSIGDTYYADS
VKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCKRFRTAAQGTDYWGQGTQVTVSSHHHHHH

pcNB2 (HER2 NB)
MEVQLVEKGGGRVQAGGSLRLRCAASGITFSINTMGWYRQAPGKQRELVALISSIGDTYYADS
VKGRFRIRRDNAKNTVYLRMRRLKPEDTAVYYCKRFRTAAQGTDYWGQGTRVTVSKHHHHH
H

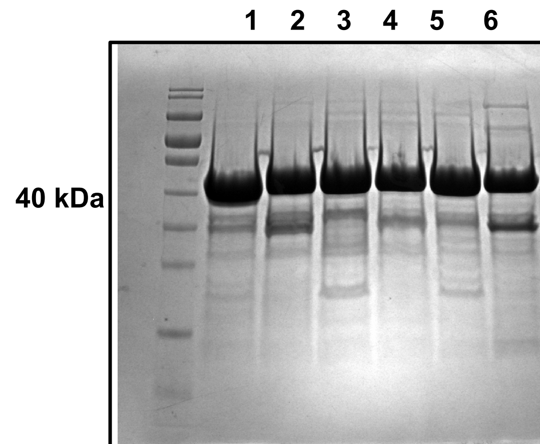
NB3 (Beta-Lac NB)
MAQVQLVESGGGSVQAGGSLRLSCTASGGSEYSYSTFSLGWFRQAPGQEREAVAAIASMGG
LTYYADSVKGRFTISRDNAKNTVTLQMNNLKPEDTAIYYCAAVRGYFMRLPSSHNFRYWGQGT
QVTVSSHHHHHH

pcNB3 (Beta-Lac NB)
MAQVQLVEKGGGKVRAGGKLRLRCTASGGSEYSYSTFSLGWFRQAPGQEREAVAAIASMGG
LTYYADSVKGRFKIKRDNAKNTVTLRMNNLKPEDTAIYYCAAVRGYFMRLPSSHNFRYWGQGT
RVTVSRHHHHHH

Figure S2. SDS-PAGE of purified Proteins. PAGE analysis of purified WT nanobodies and polycationic resurfaced mutants.



Lane 1: NB1
Lane 2: pcNB1
Lane 3: NB2
Lane 4: pcNB2
Lane 5: NB3
Lane 6: pcNB3



Lane 1: NB1-GFP
Lane 2: pcNB1-GFP
Lane 3: NB2-GFP
Lane 4: pcNB2-GFP
Lane 5: NB3-GFP
Lane 6: pcNB3-GFP

Figure S3. Brightfield images of mammalian cells. Microscopy images of 3T3 cells following treatment with 250 nM resurfaced nanobody-GFP fusions.

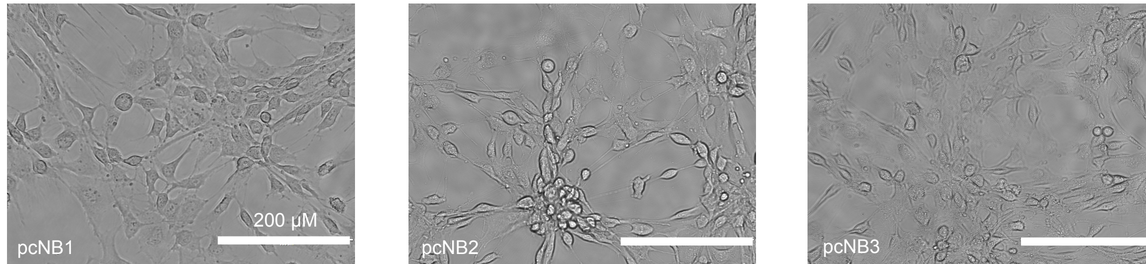
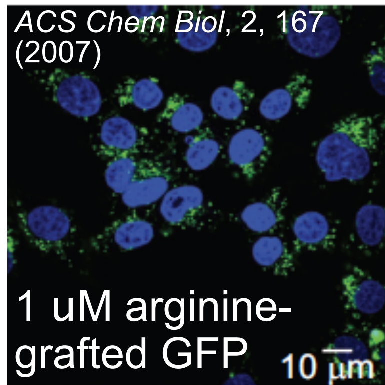


Figure S4. Fluorescent microscopy images for supercharged GFP variants.

a.



b.

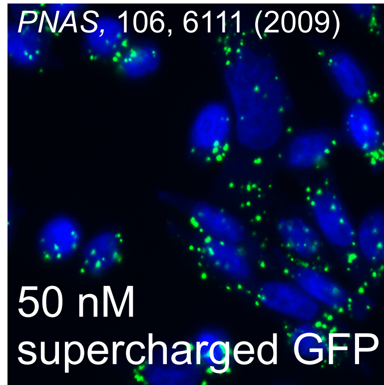


Figure S5. ITC Data. Representative ITC binding isotherms involving NB1 and pcNB1 with EGFP.