Purification of proteins for pulldown experiment with XIAP

Rationale:

XIAP is known to bind to the kinase RIPK2. RIPK2 is one of the few kinase proteins that shows overlap of reactivity to some of the compounds that we've been looking at in the context of ALK2 and other type I/ type II proteins in the BMP/TGFβ families. Therefore we were interested to see if the similarity between RIPK2 and BMP/TGFβ receptors extended into them also binding to XIAP.

Aim:

Purify the following proteins for pulldown experiments:

TGFBR1, TGFBR2, ACVR2, BMPR2, BMPR1A, BMPR1B.

Protein Purification

Proteins to be purified:

TGFBR1
MGHHHHHHSSGVDLGTENLYFQ/*SMEDPSLDRPFISEGTTLKDLIYDMTTSGSGSGLPLLVQRTIARTIVLQESIGK
GRFGEVWRGKWRGEEVAVKIFSSREERSWFREAEIYQTVMLRHENILGFIAADNKDNGTWTQLWLVSDYHEHGS
LFDYLNRYTVTVEGMIKLALSTASGLAHLHMEIVGTQGKPAIAHRDLKSKNILVKKNGTCCIADLGLAVRHDSATDTI
DIAPNHRVGTKRYMAPEVLDDSINMKHFESFKRADIYAMGLVFWEIARRCSIGGIHEDYQLPYYDLVPSDPSVEEM
RKVVCEQKLRPNIPNRWQSCEALRVMAKIMRECWYANGAARLTALRIKKTLSQLSQQEGIKM

TGFBR2
MGHHHHHHSSGVDLGTENLYFQ/*SMHNTELLPIELDTLVGKGRFAEVYKAKLKQNTSEQFETVAVKIFPYEEYAS
WKTEKDIFSDINLKHENILQFLTAEERKTELGKQYWLITAFHAKGNLQEYLTRHVISWEDLRKLGSSLARGIAHLHSD
HTPCGRPKMPIVHRDLKSSNILVKNDLTCCLCDFGLSLRLDPTLSVDDLANSGQVGTARYMAPEVLESRMNLENVE
SFKQTDVYSMALVLWEMTSRCNAVGEVKDYEPPFGSKVREHPCVESMKDNVLRDRGRPEIPSFWLNHQGIQMV
CETLTECWDHDPEARLTAQCVAERFSELEHLDRL

ACVR2
MGHHHHHHSSGVDLGTENLYFQ/*SMPLQLLEVKARGRFGCVWKAQLLNEYVAVKIFPIQDKQSWQNEYEVYSL
PGMKHENILQFIGAEKRGTSVDVDLWLITAFHEKGSLSDFLKANVVSWNELCHIAETMARGLAYLHEDIPGLKDGH
KPAISHRDIKSKNVLLKNNLTACIADFGLALKFEAGKSAGDTHGQVGTRRYMAPEVLEGAINFQRDAFLRIDMYAM
GLVLWELASRCTAADGPVDEYMLPFEEEIGQHPSLEDMQEVVVHKKKRPVLRDYWQKHAGMAMLCETIEECWD
HDAEARLSAGCVGERITQMQRLTNI

BMPR2
MEAAASEPSLDLDNLKLLELIGRGRYGAVYKGSLDERPVAVKVFSFANRQNFINEKNIYRVPLMEHDNIARFIVGDE
RVTADGRMEYLLVMEYYPNGSLCKYLSLHTSDWVSSCRLAHSVTRGLAYLHTELPRGDHYKPAISHRDLNSRNVLV
KNDGTCVISDFGLSMRLTGNRLVRPGEEDNAAISEVGTIRYMAPEVLEGAVNLRDCESALKQVDMYALGLIYWEIF
MRCTDLFPGESVPEYQMAFQTEVGNHPTFEDMQVLVSREKQRPKFPEAWKENSLAVRSLKETIEDCWDQDAEA
RLTAQCAEERMAELMMIWERNKSVSPTVNPMSTAMQNERAHHHHHH

BMPR1A
MGHHHHHHSSGVDLGTENLYFQ/*SMAFIPVGESLKDLIDQSQSSGSGSGLPLLVQRTIAKQIQMVRQVGKGRYG
EVWMGKWRGEKVAVKVFFTTEEASWFRETEIYQTVLMRHENILGFIAADIKGTGSWTQLYLITDYHENGSLYDFLK
CATLDTRALLKLAYSAACGLCHLHTEIYGTQGKPAIAHRDLKSKNILIKKNGSCCIADLGLAVKFNSDTNEVDVPLNTR
VGTKRYMAPEVLDESLNKNHFQPYIMADIYSFGLIIWEMARRCITGGIVEEYQLPYYNMVPSDPSYEDMREVVCVK
RLRPIVSNRWNSDECLRAVLKLMSECWAHNPASRLTALRIKKTLAKMVESQDVKI

BMPR1B
MGHHHHHHSSGVDLGTENLYFQ/*SMTYIPPGESLRDLIEQSQSSGSGSGLPLLVQRTIAKQIQMVKQIGKGRYGE
VWMGKWRGEKVAVKVFFTTEEASWFRETEIYQTVLMRHENILGFIAADIKGTGSWTQLYLITDYHENGSLYDYLKS
TTLDAKSMLKLAYSSVSGLCHLHTEIFSTQGKPAIAHRDLKSKNILVKKNGTCCIADLGLAVKFISDTNEVDIPPNTRV
GTKRYMPPEVLDESLNRNHFQSYIMADMYSFGLILWEVARRCVSGGIVEEYQLPYHDLVPSDPSYEDMREIVCIKKL
RPSFPNRWSSDECLRQMGKLMTECWAHNPASRLTALRVKKTLAKMSESQDIKL

/* denotes Tev cleavage site

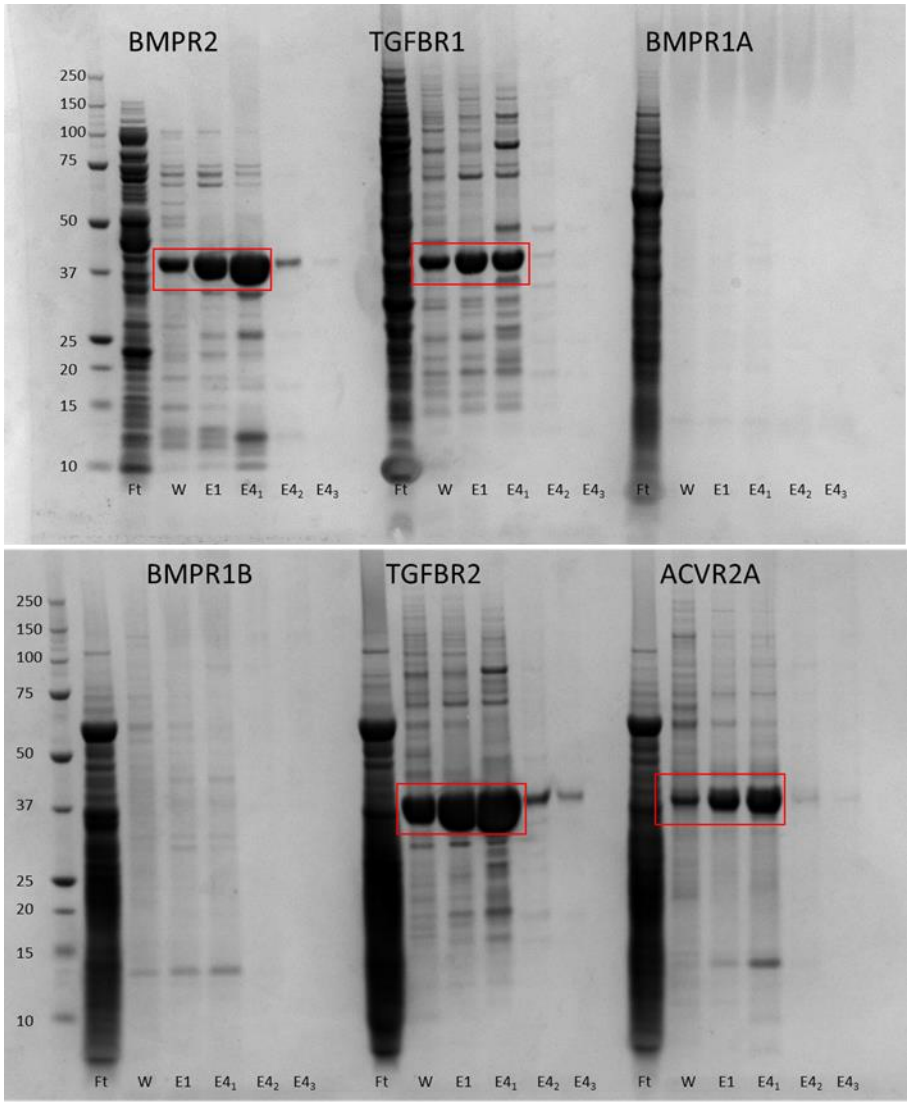
Expression:

TGFBR1, BMPR1A, BMPR1B, TGFBR2 and ACVR2 were expressed in Sf9 insect cells by Katarzyna Kupinska at 27°C for 72 hours in glass shaker flasks.

BMPR2 was expressed in *E.coli* Rossetta cells – An initial overnight culture was grown at 37C with Kanamycin. 10ml was used to inoculate 1 L LB at 37C until OD600 1.3 (accidental overgrowth), induced with 0.4mM IPTG and subsequently grown overnight at 18C before harvesting.

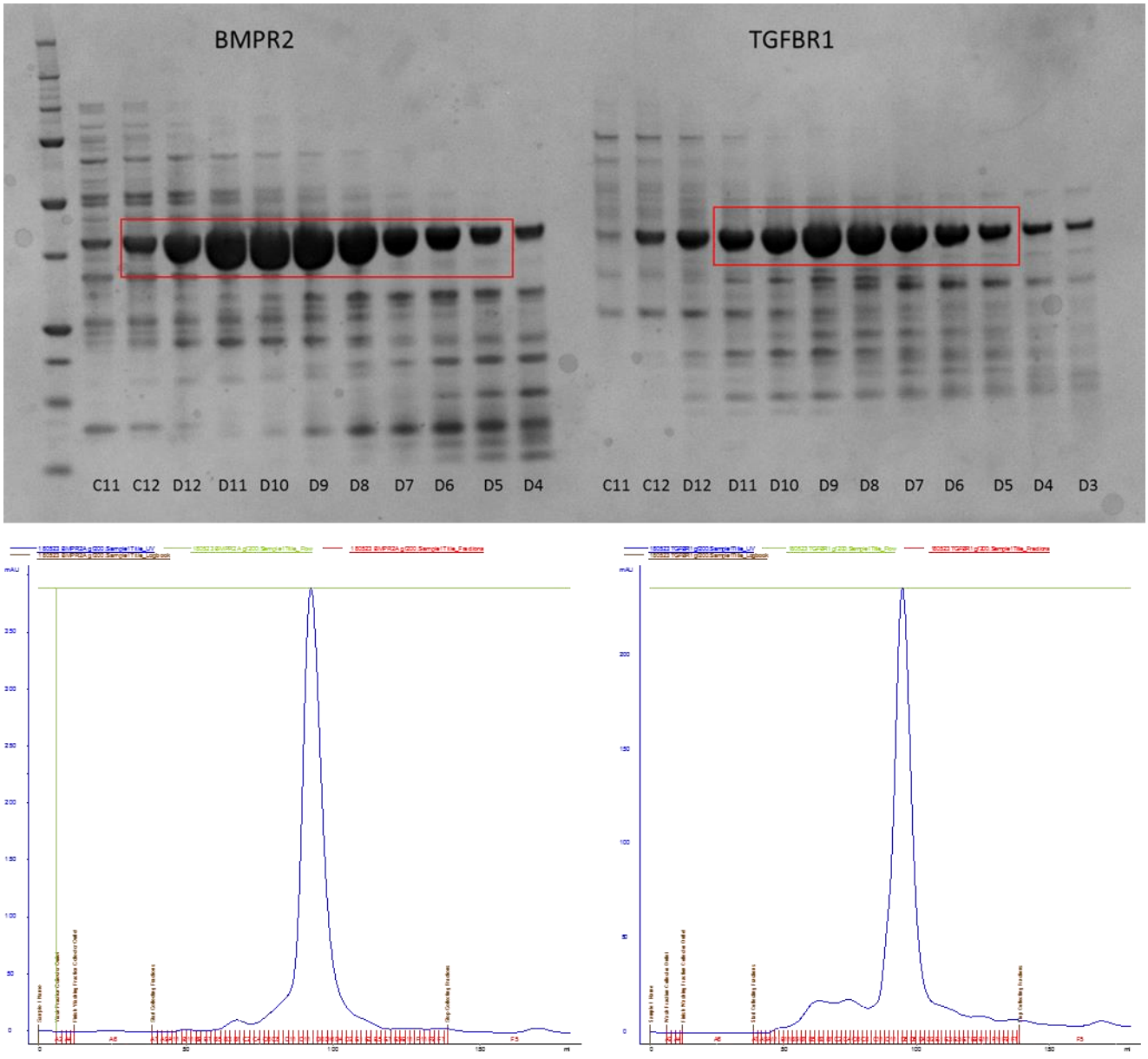
Purification:

- Thaw pellets in luke warm water
- Sonicate BMPR2A for 5 min (5 on, 10 off) on ice.
- Sonicate all other samples for 3 min (5 on, 10 off) on ice.
- Divide each sample into two centrifugation tubes and top up with 'binding buffer' (500mM NaCl, 50mM HEPES, 5mM Imidazole, 5% glycerol, ph7.5) so each tube is full (aprox 40ml per tube).
- Add 1ml PEI (5%) per tube to precipitate DNA.
- Spin at 21.5k rpm for 50 minutes.
- Incubate lysate with 2ml pre-equilibrated Ni-NTA beads at 4C for 1h. (500mM NaCl, 50mM HEPES, 5mM Imidazole, 5% glycerol, ph7.5)
- Spin down beads at 700g for 10 minutes to separate beads from lysate.
- Pour off lysate.
- Resuspend beads in 50ml binding buffer and load onto gravity flow column (collect flow through)
- Wash with 30ml wash buffer (500mM NaCl, 50mM HEPES, 30mM Imidazole, 5% glycerol, ph7.5).
- Elute in 7ml elution buffer 1 (500mM NaCl, 50mM HEPES, 50mM Imidazole, 5% glycerol, ph7.5).
- Elute in 3 x 7ml elution buffer 4 (500mM NaCl, 50mM HEPES, 250mM Imidazole, 5% glycerol, ph7.5).
- Add 1mM TCEP to all fractions.
- Run samples on an SDS PAGE gel (mix 5ul of loading dye with 15ul sample, boil for 3 minutes and load 10ul onto the gel.) and run at 160V for 50 minutes.
- Pool elution fractions E1 - E4(3)
- Add Tev protease to fractions which contain protein (Wash 2, E1, $E4_1$)
- Incubate at 4C overnight.

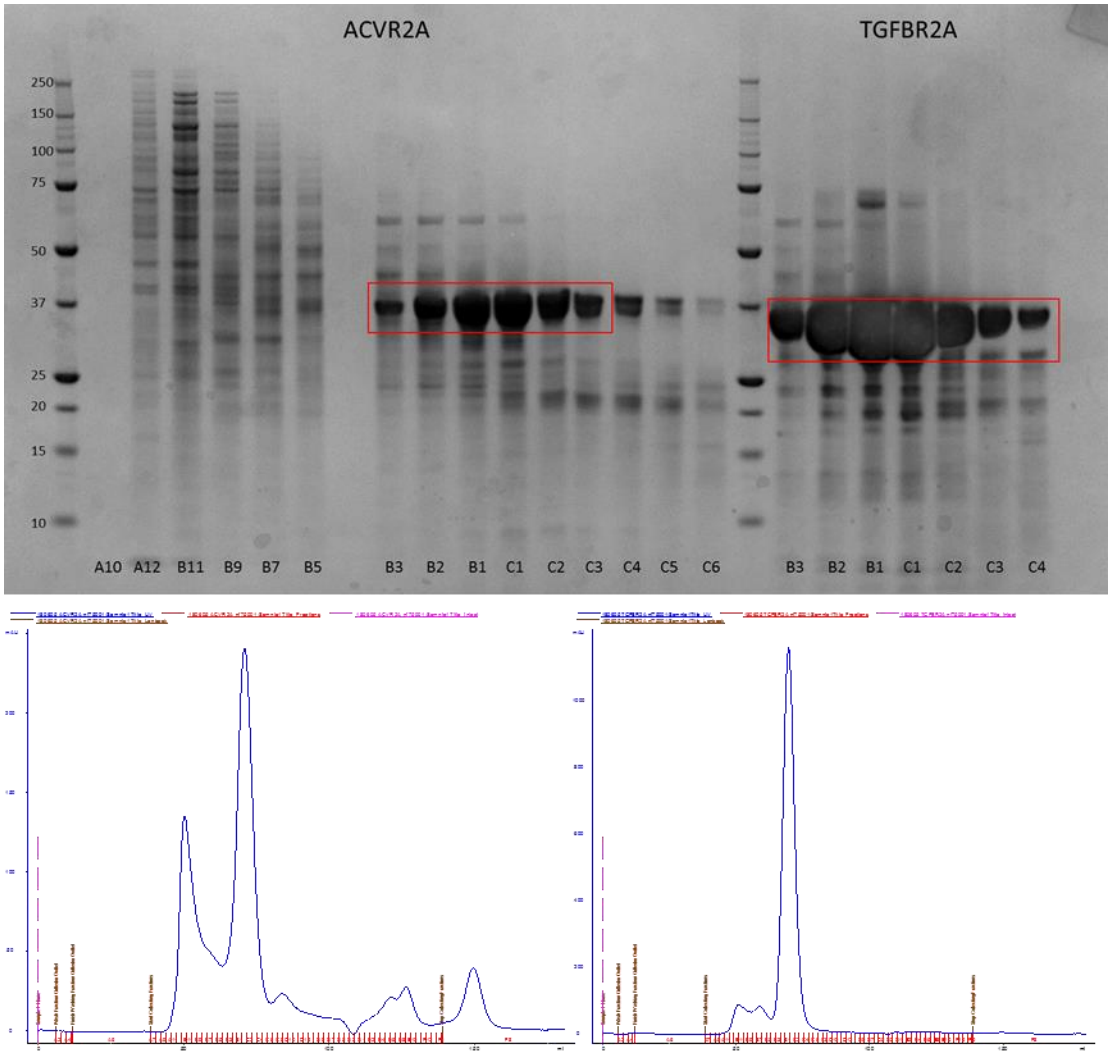


Samples corresponding to the nickle column fractions run on an SDS PAGE – FT (flowthrough combined with binding buffer wash), W (wash with wash buffer), E1 (elution with E1 elution buffer), E4$_{(1-3)}$ (three elution's with elution 4 buffer)

- Concentrate down boxed samples to <5ml and run on a pre-equilibrated GF 200 column (BMPR2 and TGFBR1) or a GF 75 column (ACVR2 or TGFBR2) using standard gel filtration buffer (300mM NaCl, 50mM HEPES, 5mM Imidazole, 5% glycerol, ph7.5).
- Collect fractions and run a gel of peak.



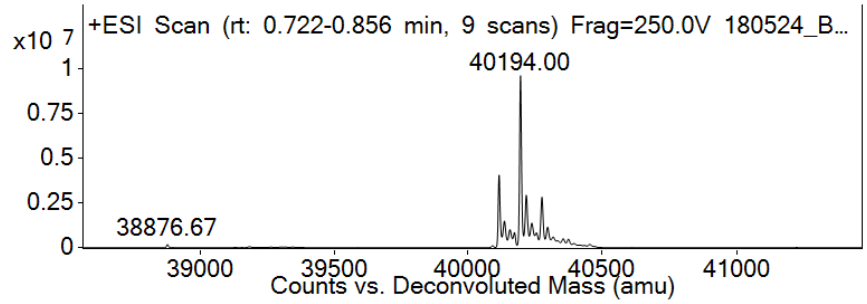
BMPR2 and TGFBR1 SDS PAGE gel (top) shows samples from the relevant size exclusion column fractions, corresponding to the UV peak shown in the bottom panel.



ACVR2 and TGFBR2 SDS PAGE gel (top) shows samples from the relevant size exclusion column fractions, corresponding to the UV peak shown in the bottom panel.

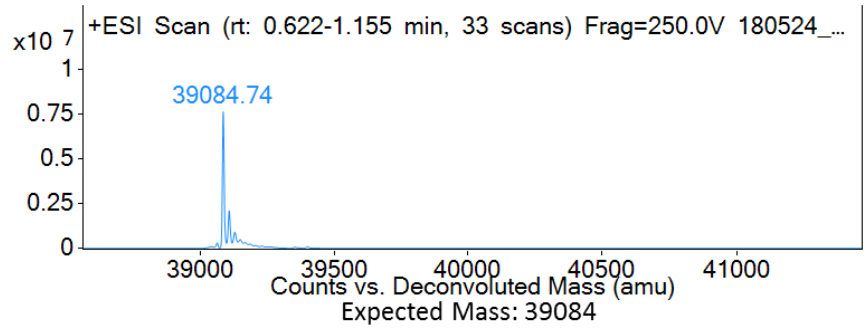
- Mass spec proteins.
- Pool fractions back together and concentrate down before flash freezing in liquid nitrogen.

BMPR2



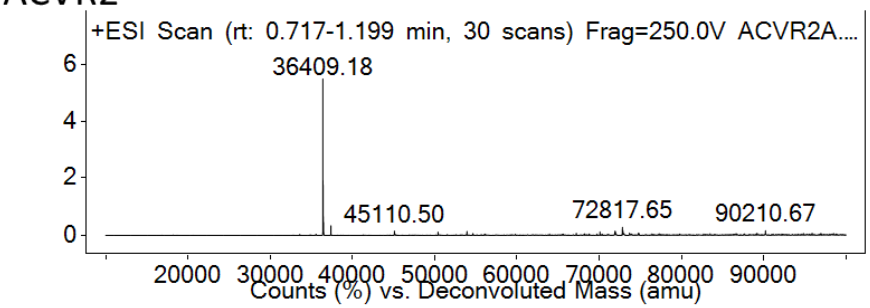
Expected Mass: 40113.7 = +80 phosphorylation

TGFBR1



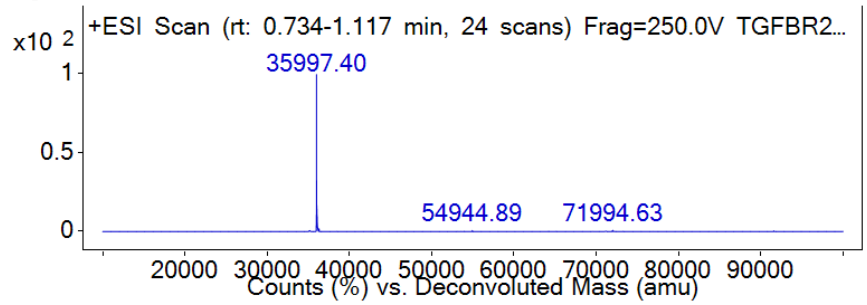
Expected Mass: 39084

ACVR2



Expected Mass: 36497.9 – Met+ acet = 36408

TGFBR2



Expected Mass: 35972.1 (unexplained 20 Da difference)

Mass spec of purified proteins.