Tuesday, October 18, 2004

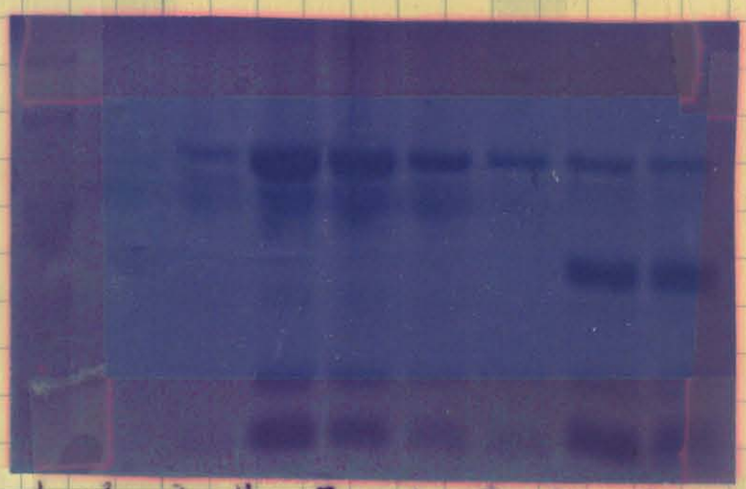
- The 16-mL GST-purified protein solution in TBS was incubated with 1.4 mL of FLAG-affinity resin (used 1× previously) w/ 40 µL of protease inhibitor cocktail at rt for 1.5 hours (then stored in fridge for 1 hour while at a meeting.)

- The resin was filtered, washed with 80 mL TBS, and then eluted with 1 to 1.2 mL aliquots of 0.1 M glycine, pH 3.5 collected into 40 µL of 500 mM Tris·HCl, pH 8.0. The concentration of protein/sample was determined using the Biorad protein assay. All fractions were stored at 4°C.

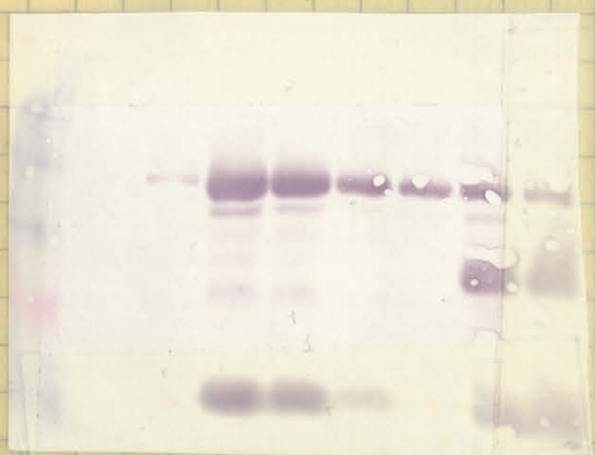
Wednesday, Oct. 19, 2004

TEV cleavage (2 rxns run currently)

- Fraction 3 (600 µg in 1.1 mL) was split into 2 portions of 300 µg GST-ENLYFQC-Pax(38-557)-FLAG in 550 µL elution buffer/Tris. The following rxn was incubated at 37°C for 4 hours:
  - ≈ 300 µg protein in 550 µL
  - 60 µL of 500 mM Tris·HCl, pH 8.0
  - 15 µL mTEVp



1 2 3 4 5 6 7 8 9



1 2 3 4 5 6 7 8 9

1° 1.5 µL/10 mL TBST anti-FLAG ab

In 2 FLAG elution 1 (↓ Biorad quantification)
3 FLAG el. 2 (60 µg/mL)
4 FLAG el. 3 (600 µg/mL)
5 FLAG el. 4 (400 µg/mL; 1.1 mL)
6 FLAG el. 5 (180 µg/mL; 1.1 mL)
7 FLAG el. 6
8 TEV cleavage A, t = 4 h
9 TEV cleavage B, t = 4 h

- The mTEVp cleavage reactions were stored at 4°C overnight, then used in ligation reactions.

EPL: pY31 Wednesday, Oct. 19, 2004

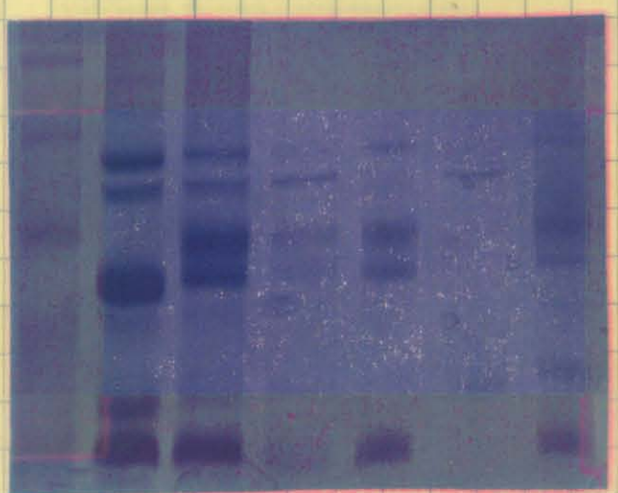
- 200 µg of Cys(Pax38-557)-FLAG (from TEVp cleavage A on p. 65) in ≈ 600 µL of mTEVp cleavage mixture was concentrated to 60 µL using a 50-kDa MWCO centrifugal filter.

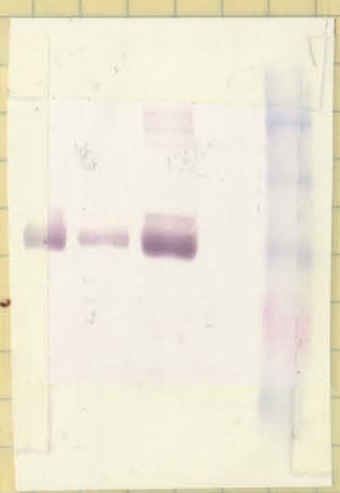
- 10 µL were removed for gel analysis. The remaining 50 µL were added to 250 µg of lyophilized AcHis6-Pax(2-36, pY31)-SBn and aliquots were removed and reacted as follows:

AII: 20 µL of protein/thioester, 10 µL of 50 mM Tris, 300 mM NaCl, pH 8.0, 2 µL of 1 M MESNA. (No precip. after 24 h)

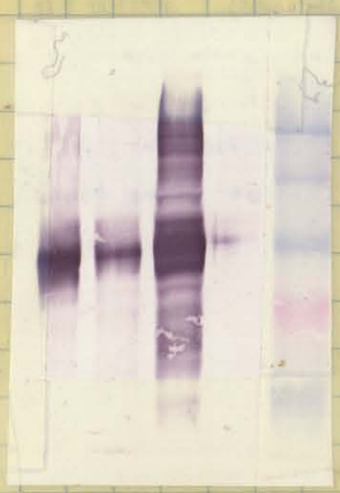
AIII: 10 µL protein/thioester soln., 10 µL of 200 mM MES, 300 mM NaCl, pH 6.1, 2 µL of 1 M MESNA (100 mM final concentration) (A ~~tiny~~ amount of precipitation after 24 h.)

AIII: 10 µL protein/thioester, 10 µL of 200 mM MES, 300 mM NaCl, pH 6.1, 0.8 µL of 1/20 dil. of thiophenol (0.2%) (some precip. after 24 h.)





1° ab anti-His 6 (5 µL in 10 mL TBST)



1° ab rabbit anti pY31-Pax (1.5 µL in 10 mL TBST)

In 1 benchmark ladder
2 10 µL Cys-Pax(38-557)-FLAG
3 rxn A, t = 24 h
4 rxn AII, t = 24h, supernatant only (a tiny amnt of pellet got taken up also)
5 " , t = 24 h, 1/2 of pellet resuspended
6 rxn AIII, t = 24h, supernatant only
7 " , t = 24 h, 1/2 of pellet resuspended

(In each reaction ≈ 2.5 mg/mL thioester. - 0.5 mM)