Phosphorylation of GST-Pax(38-557)-FLAG and AC-His x6-Pax(2-557)-FLAG

Wednesday, July 27, 2005

Substrates used:

GTP : Elution 4 fr. p.137 of GST-Pax(38-557)-FLAG : 265 ug/mL, 3.77 uL/ug

LigP : 1/3 dil. of NCL product from p.136 (assuming 1.5 mg/mL original concentration from an ideal [] of 3) : 500 ug/mL, 2 uL/ug ACH x6-Pax(2-557)-FLAG

- Src, ERK, and JNK2 (Biosource phosphorylation) ↑↑ JNK 2 concentration
  3 40-uL reactions were run w/ 2 ug (7.6 uL) GTP
  2 40-uL reactions were run w/ 2 ug (4 uL) LigP :

  2 ug substrate (ie. 7.6 uL)
  8 uL of 1 mM ATP (diluted from 10 mM in TBS) final [] = 200 uM
  2.25 uL of 226 mM MgCl2 (diluted in TBS) final [] = 15 mM
  1.5 uL Src (450 units), or 4 uL ERK (148 units), or 7 uL JNK2
  TBS to 40 uL total (20.65, 18.15, and 15.15 for Src, ERK, +JNK2)

  The above reagents were combined in a 0.65 mL eppendorf, pipetted 7 x to mix, flicked once and incubated at 30°C for 15 min. 40 uL of 2 x SDS reducing gel loading buffer was added for a final substrate concentration of 25 ng/uL. A 1/5 dil. (10 uL in 40 uL 1x loading buffer) was made to give a 5 ng/uL solution.

- JNK1 (upstate) phosphorylation of GST-Pax(38-557)-FLAG (GTP)
  2 50-uL reactions :

  2 ug substrate (7.6 uL)
  8 uL of 1mM ATP
  2.25 uL of 226 mM MgCl2
  4 uL of 0.1% Brij
  4 uL of 10 mM dtt
  4 uL of 50 mM EGTA
  TBS to 50 uL total (10.15 or 15.15 uL)
  5 uL (0.5 ug) or 10 uL (1 ug) of JNK1α1

  The mixtures were pipetted 7 times, flicked, then incubated at 30°C for 15 min. 30 uL of 2 x loading buffer were added (to give 25 ng/uL) and a 1/5 dilution was made (to give 5 uL/uL).

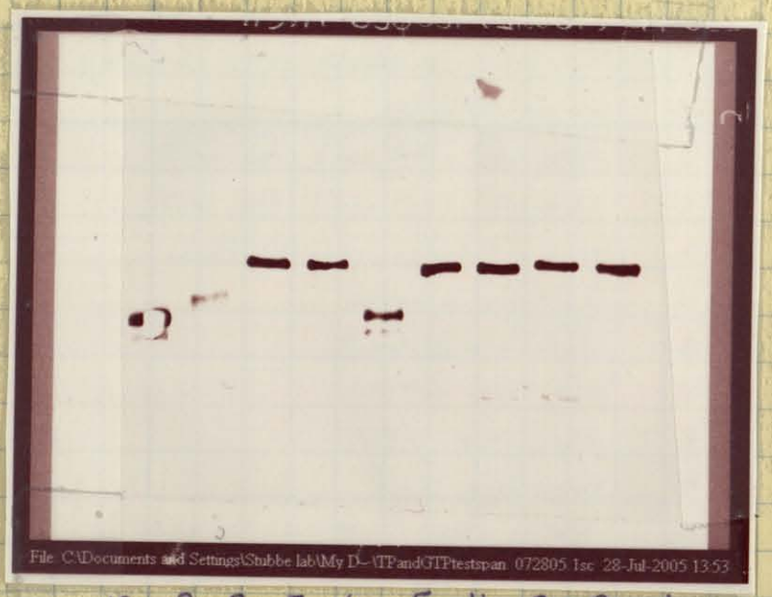
Chemiluminescent gels from p. 138 phosphorylation (pan, pY31, and pY118)

Thursday, July 29, 2005

1° mouse anti-paxillin (1/1,000)
2° goat anti-mouse HRP (1/10,000)
exposed for 4 min.

GTP = GST-Pax(38-557)-FLAG
LigP = AcHisx6-Pax(2-557)-FLAG

In		
	1	7 μL ladder
	2	JNK1α1 (0.5 mg kinase in rxn)
	3	JNK1α1 (1.0 mg kinase in rxn)
	4	JNK2 (7 μL!) for GTP
	5	GTP - no kinase
	6	LigP - no kinase
	7	~~ERK~~ for GTP Src
	8	ERK for GTP
	9	Src for LigP
	10	ERK for LigP

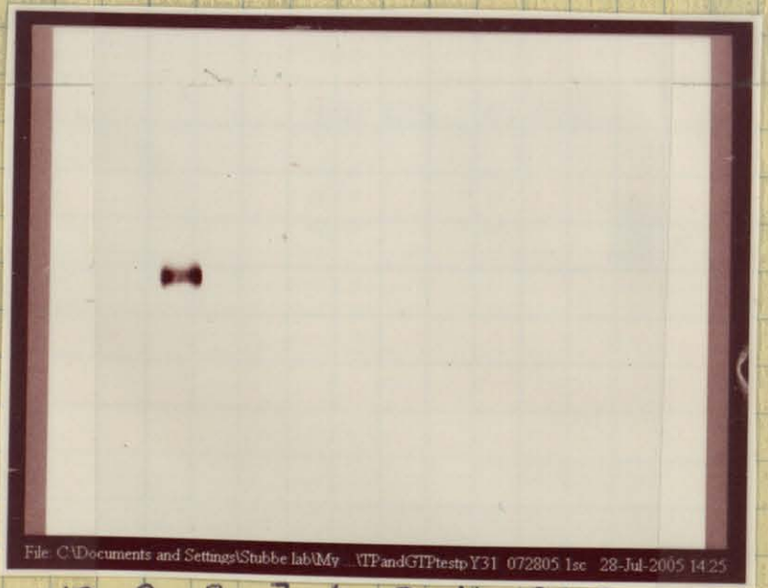


10 9 8 7 6 5 4 3 2 1

6 μL (of 1/5 dilution) - 30 ng loaded/well

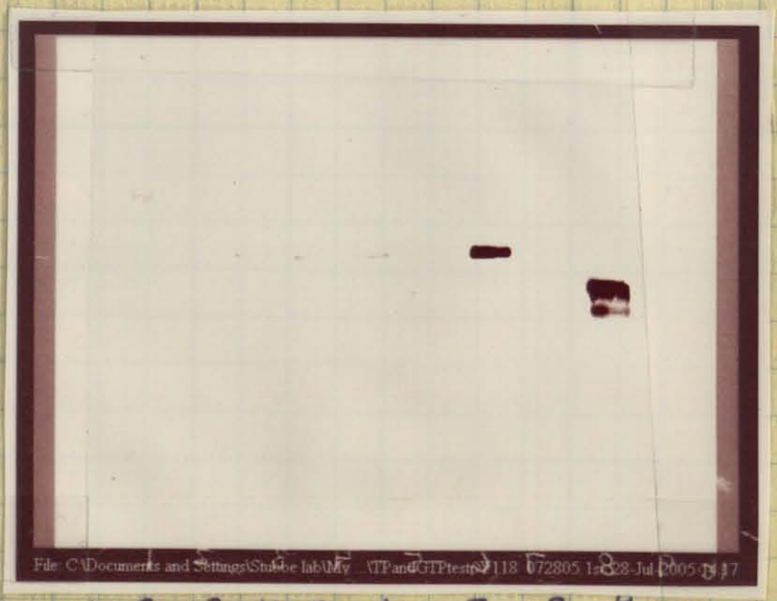
30 ng loaded for anti-pax, pY31 and pY118
250 ng loaded for anti-pS128 + pS178

1° rabbit anti-pY31 pax (1/2,000)
2° goat anti-rabbit HRP (1/20,000)
exposed for 5 min.



10 9 8 7 6 5 4 3 2 1

1° rabbit anti-pY118 pax (1/2,000)
2° goat anti-rabbit HRP (1/20,000)
exposed for 3 minutes



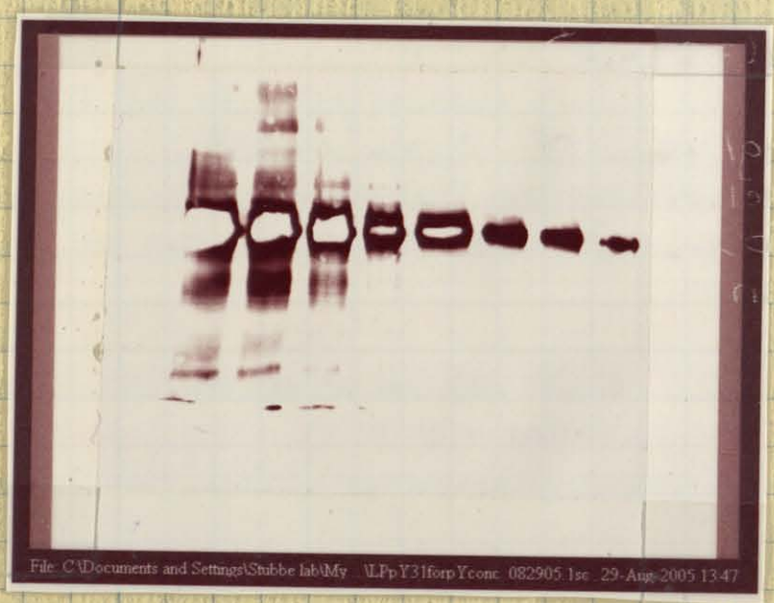
1 2 3 4 5 6 7 8 9 10

30 ng (6 μL of a 1/5 dilution) loaded per well

Chemiluminescent detection of pY31 Native Chem. Lig. product

Monday, August 29

1° rabbit anti-pY31 pax (1,2,000)
2° goat anti-rabbit HRP (1,20,000)



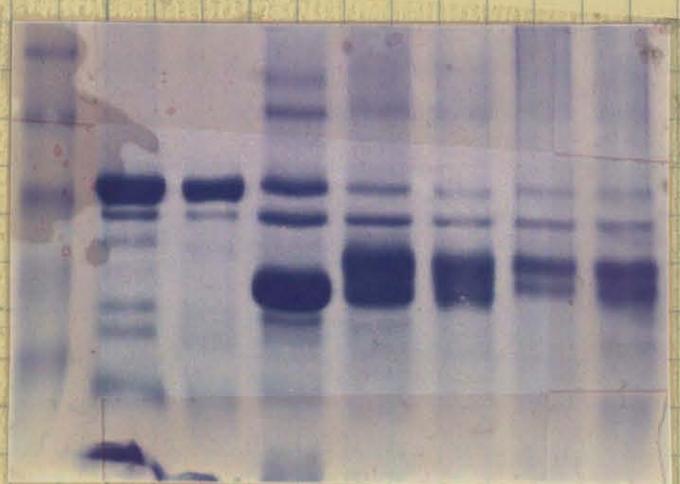
9 8 7 6 5 4 3 2 1

expore time = 30 seconds
run on a 0.75-width gel

ln 1	7 μL protein benchmark
2	3 μL pY31 B (1/5 of A)
3	6 μL pY31 B
4	12 μL pY31 B
5	24 μL pY31 B
6	5 μL pY31 soln. A
7	10 μL pY31 A
8	15 μL pY31 A
9	20 μL pY31 A

It appears that loading between 5 and 10 μL of the pY31 sample B results in the best visualization.

Stock gel solutions for pY31 paxillin
130 μL (of the 300 μL total on p.146) with 130 μL 2× SDS page



1 2 3 4 5 6 7 8

ln 1	ladder
2	30 μL FLAG (stock soln.)
3	before ligation (8 μL + 22/30)
4	15 μL FLAG
5	NCL w/ pY31 (30 μL)
6	NCL w/ pY31 (30 15 μL)
7	NCL Y31 (7/20)
8	NCL glycerol stock