Generation of Phosphorylated Protein Standards for 2D Gel Western Blotting

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Talk Outline

- Kinases and their importance
- Anti-phosphoprotein Western blotting
- ^{32}P labeling with recombinant serine kinase
  - Interpretation of results
- ^{32}P labeling with recombinant tyrosine kinase
- Conclusions, Collaborators

About Protein Kinases:

- The human genome contains about 500 protein kinases:
  ~410 are serine/threonine kinases
  ~90 are tyrosine kinases
  most are not characterized
- Rough estimates suggest that 30-50% of proteins in mammalian cell lysates are serine/threonine phosphorylated. Tyrosine phosphorylation is less common.
- Kinases are important in cancer mechanisms

Cancer is like a Runaway Car

- Mutated kinases act as the gas pedal stuck to the floor
- Inactivated tumor suppressor proteins (e.g. p53) are defective brakes
- Up-regulated telomerase provides endless gas by maintaining telomeres

Robert Weinberg, Biology of Cancer, 2007 p383

Pharmaceutical companies are very interested in kinase inhibitors

❖ Gleevec (Norvartis), approved in 2001, was the first and is used in treating chronic myelogenous leukemia (CML) - a miracle drug.

❖As of 11/1/10,Wikipedia shows 19 tyrosine kinase inhibitors that have reached clinical trials, although not all have been approved.

❖ www.Prokinase.biz has 158 kinase inhibitors for sale at modest prices including 21 MAP kinase inhibitors.

Unfortunately, cancers evolve and stop responding to KIs

- It's difficult to say which kinase pathways are turned on or off. Many labs are working on this from a lot of angles.
- Klabs has optimized 2DE Western blotting for phosphoproteins as a tool to aid this research. We can see kinase *substrates* that are difficult to detect by other methods.

Standardized 2DE Western blotting with anti-phosphoamino acid antibodies to find important phosphorylated proteins (kinase substrates)

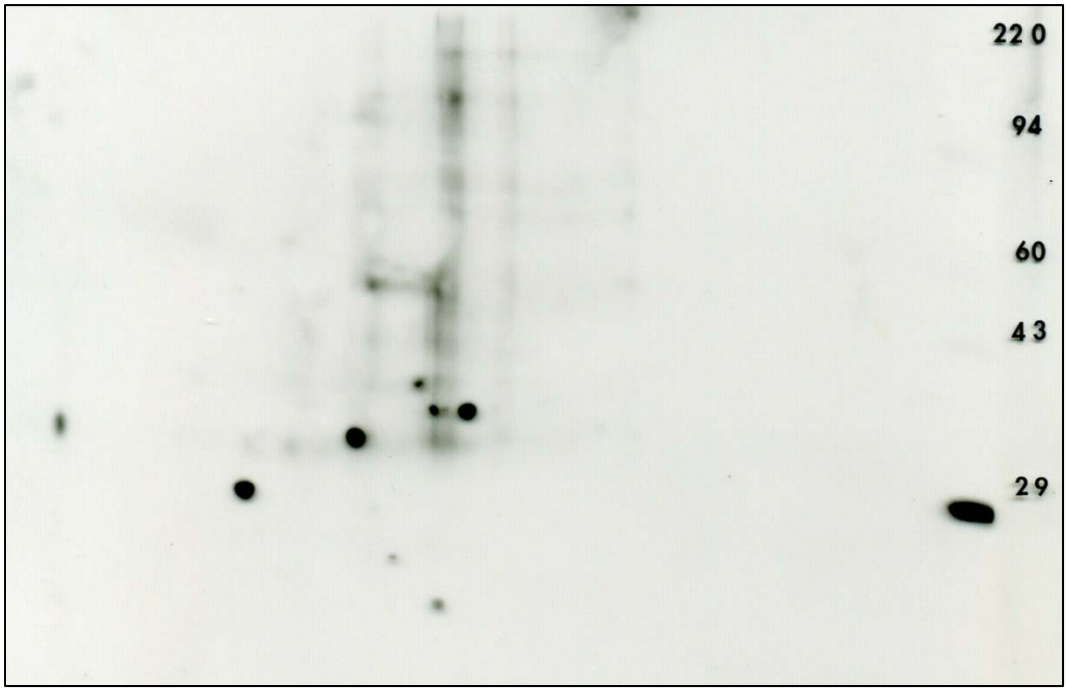
- A 2D gel is run, the proteins transferred to PVDF, the membrane shaken overnight with an antibody, and then treated with ECL or ECL Plus before exposure to x-ray film

- The antibody is either a monoclonal antibody,PY20, against phosphotyrosine or mixed Qiagen monoclonal antibodies, Q5 (anti-phosphoserine) + Q7 (anti-phosphothreonine)

- The ECL film pattern is matched to the stained PVDF blot which is matched to a duplicate Coomassie gel for spot-cutting for mass spectrometry.

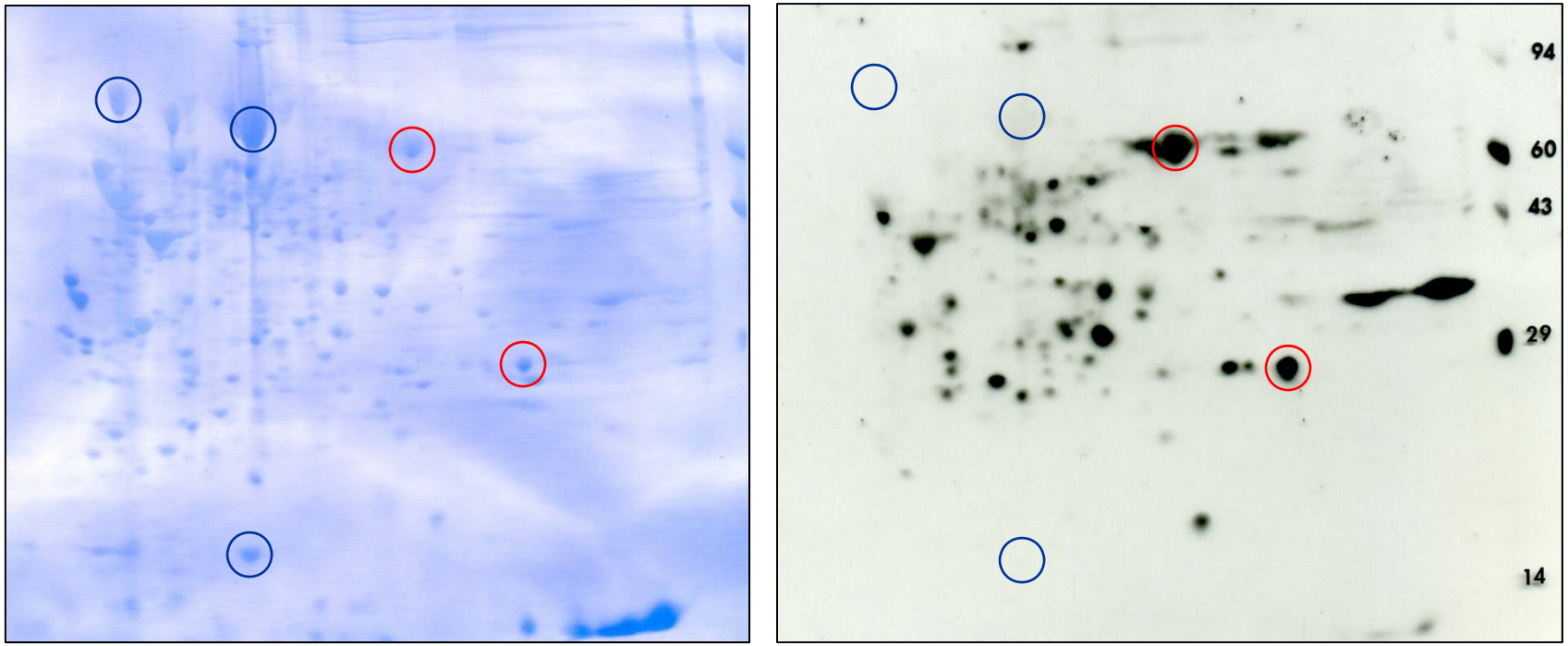
More method details at:
www.kendricklabs.com

Example of Anti-phosphotyrosine 2D Western blot using the PY20 antibody



Mouse embryonic fibroblast after siRNA knockdown (shown with permission). The 29 kDa MW marker, carbonic anhydrase, is probably phosphorylated, we're not sure. ***It would be useful to have an internal standard known to be phosphorylated.***

Example of Anti-phosphoserine 2D Western blot using the Qiagen Q5 antibody.



Left: Coomassie blue stained PVDF transblot from 2D gel run with rat liver homogenate. *Right:* Anti-PSer Western film pattern from the left blot. Some abundant proteins light up (red circles) and some don't (blue). We're not sure how much nonspecific binding occurs. *Having internal standards would be helpful here too.*

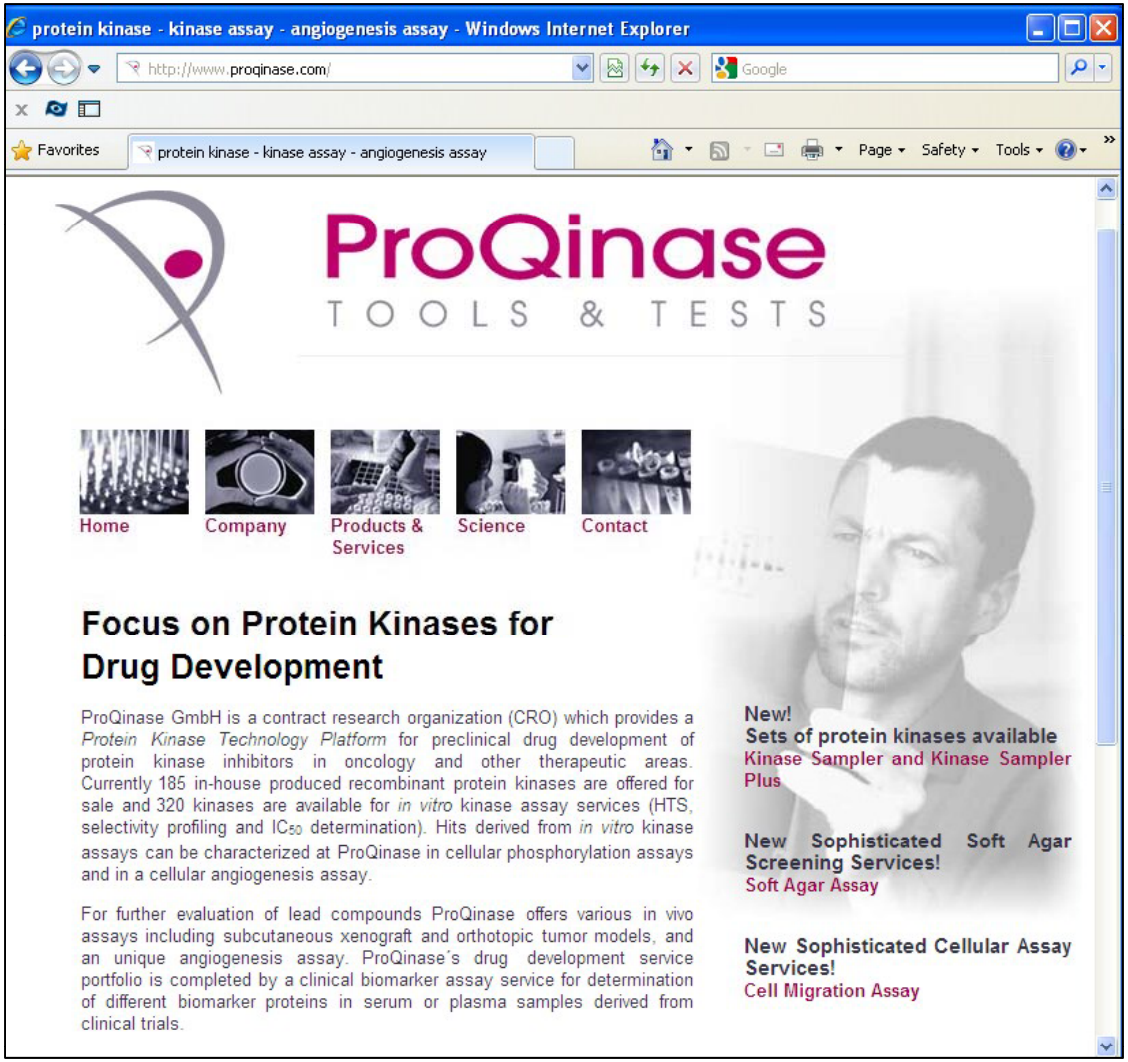
Surprisingly, phosphorylated protein standards aren't commercially available. (Mass spectrometry labs use phosphorylated *peptides.*)

So we decided to make our own phosphorylated standards using recombinant kinases and substrates.

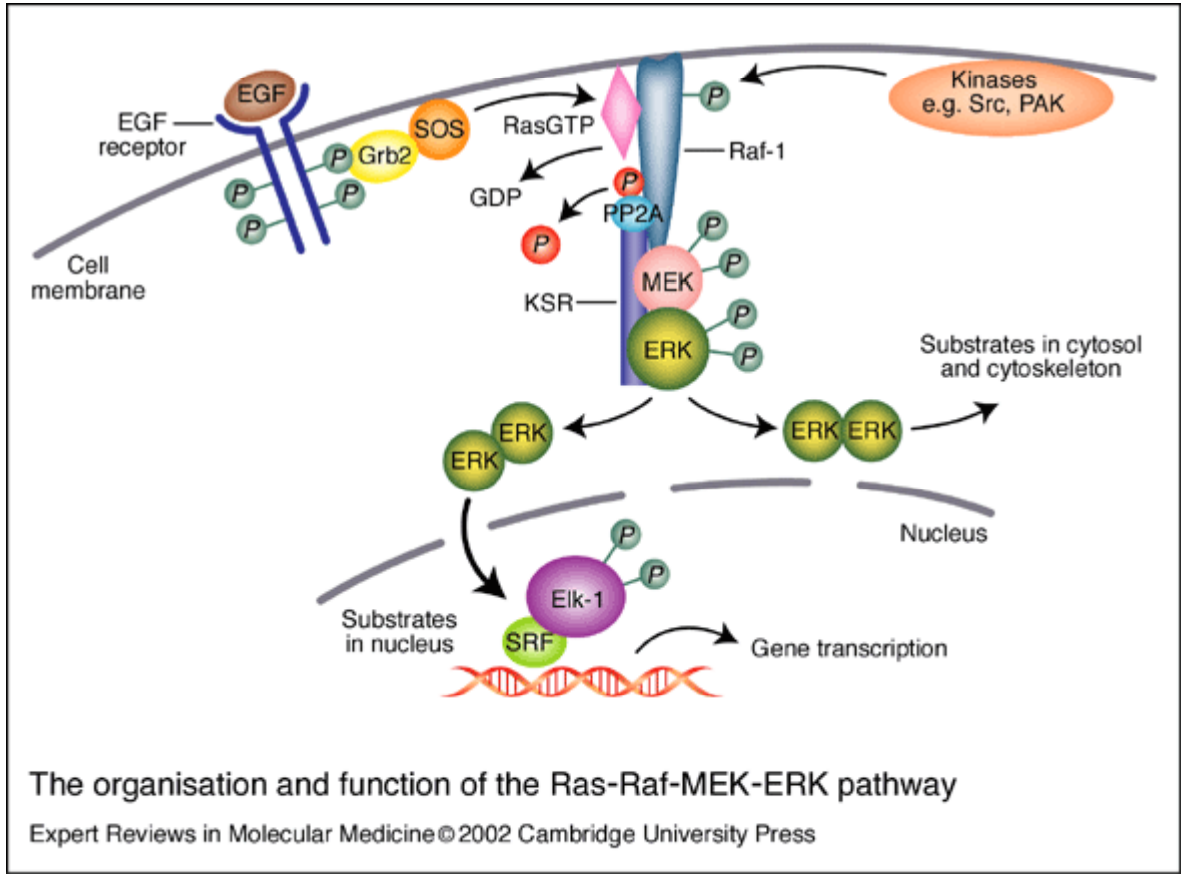
New company called ProQinase!

❖ specializes in recombinant protein kinases

❖ based in Freiburg, Germany but has US distributor



Recombinant MEK kinase and ERK substrate were purchased from ProQinase.



MEK is a phosphoserine kinase involved in signal transduction.

The phosphorylation reaction was carried out in vitro

MEK-1 kinase (MW 43,600 from Sf9 insect cells)

ERK-2 substrate (MW 45,500 from *E. coli*)

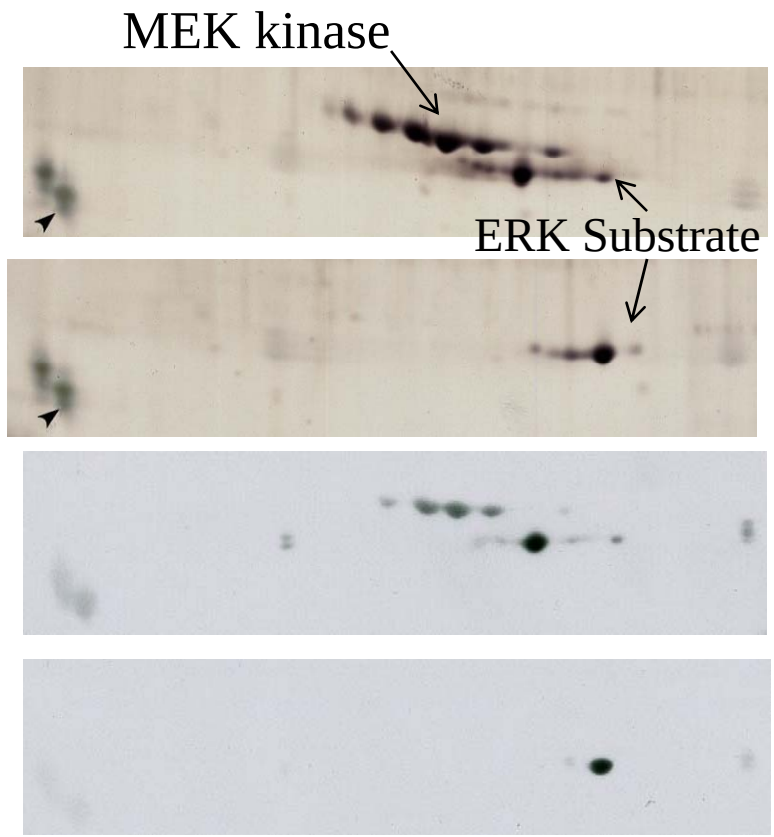
ProQinase Standard Assay Buffer

Either cold ATP or ^{32}P-labeled ATP

The reaction was allowed to proceed 40 min at 30^{o} C, stopped with H_3P0_4, diluted with urea buffer and aliquots run on 2D gels.

2D Silver stain versus Western blot

MEK + ERK + PO_4	silver
ERK + PO_4	silver
MEK + ERK + PO_4	PSer WB
ERK + PO_4	PSer WB

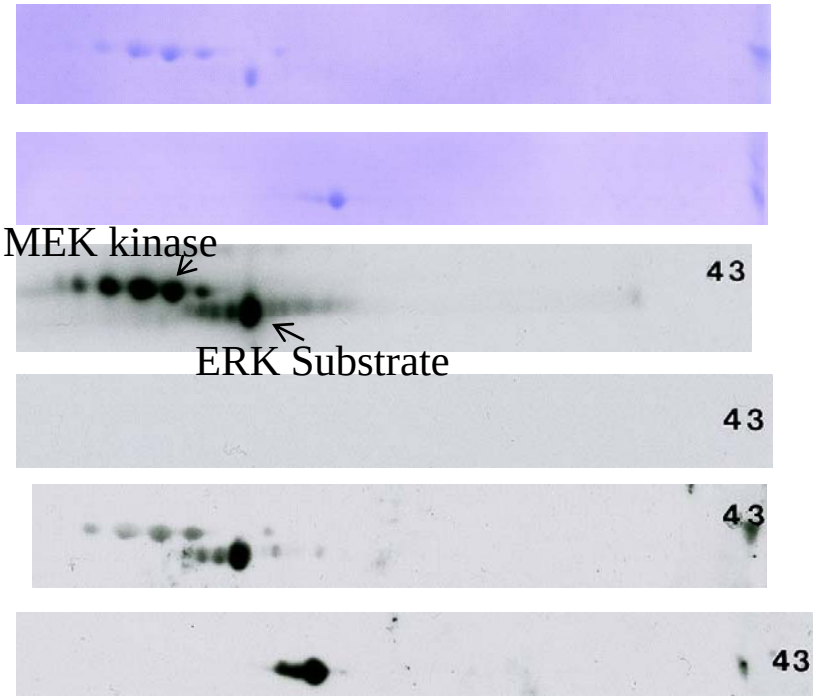


Aligned 2D gel sections

When the MEK kinase is present the main ERK isoform moves in the acidic direction during IEF; this suggests phosphorylation has occurred as expected. But surprisingly, the anti-PSer ab also lights up the MEK kinase and *both* forms of the ERK substrate. What's going on?

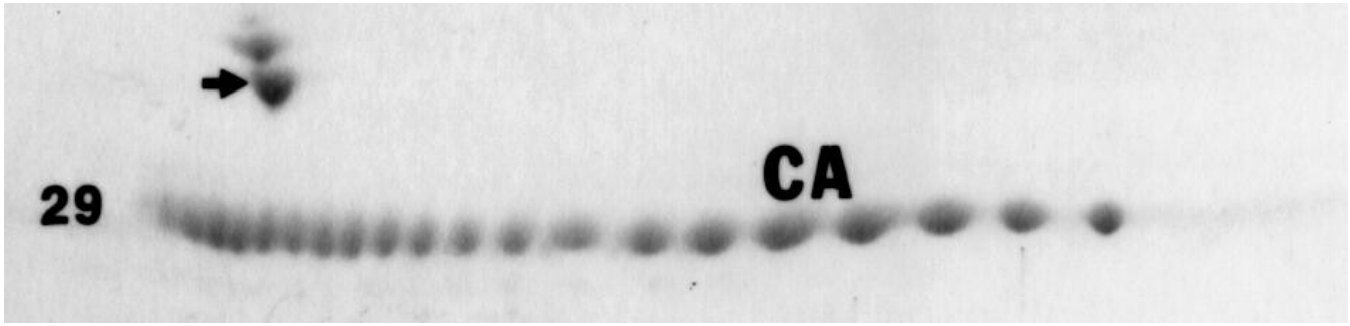
Coomassie and WB versus ^{32}P label

MEK + ERK + $^{32}PO_4$	CB-PVDF
ERK + $^{32}PO_4$	CB-PVDF
MEK + ERK + $^{32}PO_4$	x-ray film
ERK + $^{32}PO_4$	x-ray film
MEK + ERK + $^{32}PO_4$	ECL-WB
ERK + $^{32}PO_4$	ECL-WB



Two 2D gels were run: MEK kinase + ERK substrate + ^{32}P, and substrate alone + ^{32}P. The blots were exposed to x-ray film to show radiolabeled proteins, and then anti-PSerine WB was carried out using ECL. *Conclusions: The MEK **kinase** and ERK substrate are both clearly phosphorylated. The unphosphorylated ERK substrate lights up with the anti-PSer antibody suggesting non-specific binding. (Unless ERK is phosphorylated by the insect cell line.)*

Carbamylated proteins provide evidence that 2D gels show single charge changes

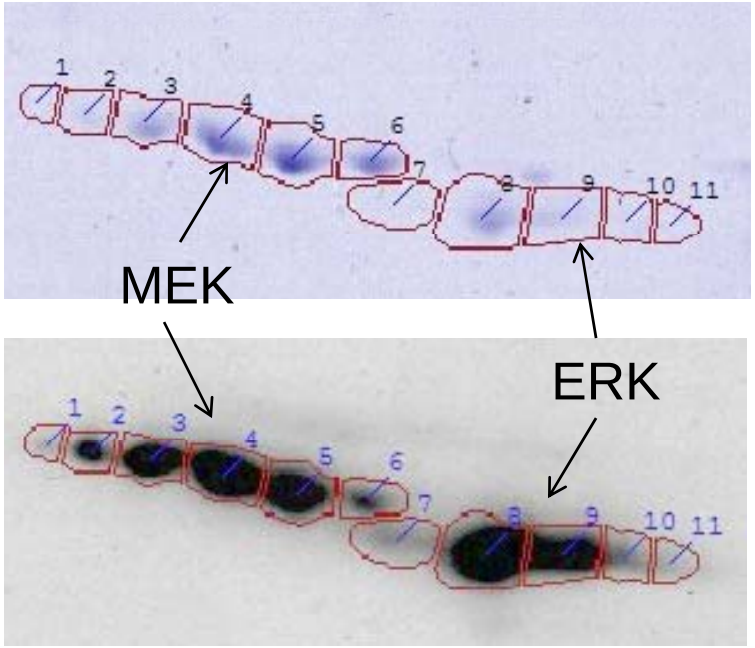


Carbamylated carbonic anhydrase standard shows a train of spots, each differing by a single charge as lysine groups are progressively blocked. The arrow points to our internal standard tropomyosin.

NL Anderson and BJ Hickman, *Anal Biochem*, *1979*; 93: 312.

Quantitative Analysis

	Spot #	CB Vol	Film Vol	Film/CB Specific Activity	Ratio to most basic
MEK	1	0.5	3	7	4
	2	1.1	32	28	14
	3	6	110	17	9
	4	17	156	9	5
	5	21	100	5	3
	6	10	18	2	1
ERK	7	1.1	14	12	1
	8	10	232	23	2
	9	3	78	28	3
	10	1.1	12	11	1
	11	0.4	3	10	1



^{32}P-labeled MEK/ERK was run on a Coomassie-stained 2D gel and exposed to x-ray film. Both gel and film were quantitatively scanned and analyzed with Progenesis SameSpots software from Nonlinear Dynamics. But results didn't make sense in terms of phosphate groups per charge isoform.

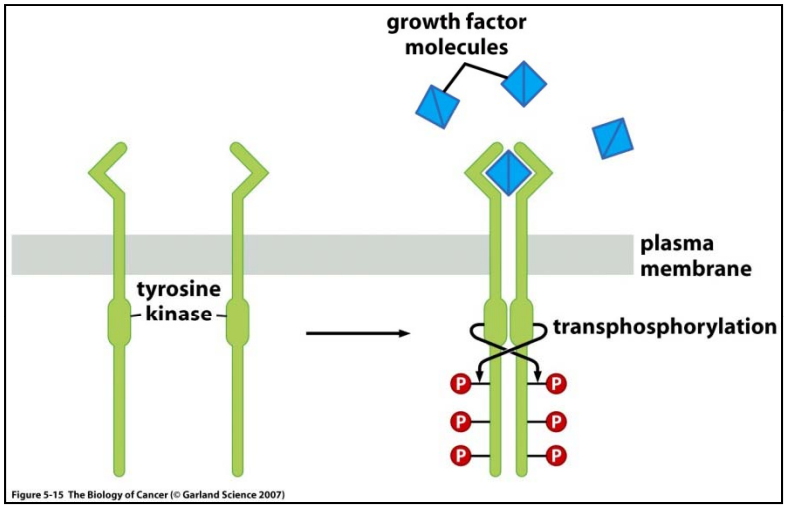
First assumption, that each aa site reaches 100% phosphorylation before the next one is filled, is naive.

MEK	% AA phosphorylation			
Condition 1	0%	0%	100%	0%
10 molecules	aa 12	aa 48	aa 96	aa 192
1			P-Ser	
2			P-Ser	
3			P-Ser	
4			P-Ser	
5			P-Ser	
6			P-Ser	
7			P-Ser	
8			P-Ser	
9			P-Ser	
10			P-Ser	

In reality... variable phosphorylation of each aa site. We'll have to send the individual spots to our collaborator at Columbia University Protein Core for analysis to determine phosphates per spot isoform.

MEK	% AA phosphorylation			
Condition 4	10%	30%	60%	90%
10 molecules	aa 12	aa 48	aa 96	aa 192
1	P-Ser	P-Ser	P-Ser	P-Ser
2	20%	P-Ser	P-Ser	P-Ser
3		P-Ser	P-Ser	P-Ser
4		30%	P-Ser	P-Ser
5			P-Ser	P-Ser
6			P-Ser	P-Ser
7			30%	P-Ser
8				P-Ser
9				P-Ser
10				10%

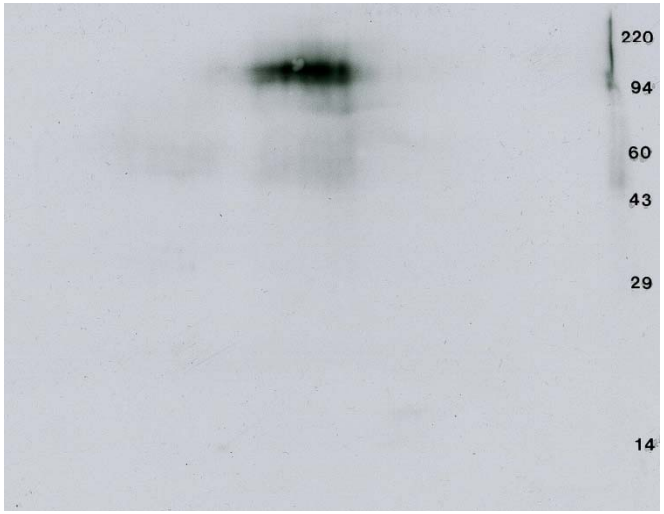
Vascular Endothelial Growth Factor Receptor (VEGF)



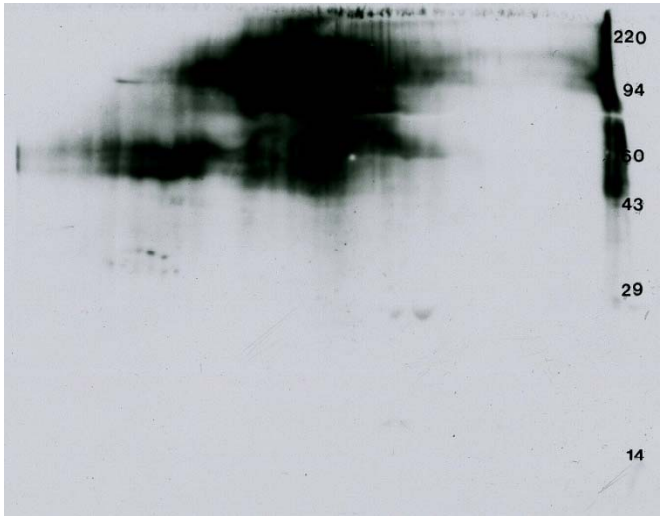
VEGF is a transmembrane receptor

- VEGF stimulates the formation of a new blood supply for developing tumors via it's receptor. Inhibitors of VEGF-R (Avastin/Genentech) have been approved to treat a variety of cancers.
- We bought VEGF-R from ProQinase, ^{32}P labeled assuming self-phosphorylation, TCA ppt'd to concentrate, ran a 2D gel, transferred to PVDF, exposed 4 days and then did an anti-PTyr Western blot.

The PY20 antibody is *very* sensitive!



^{32}P Autorad: 4 day x-ray film exposure to show ^{32}P labeled protein. Nothing was visible by Coomassie blue on the PVDF.



Western blot: 30 sec film exposure after anti-PTyr antibody exposure and ECL treatment.

We'll buy and label a non-receptor tyrosine kinase to use as an internal standard.

Conclusions

- Generating phosphoylated protein standards using recombinant proteins from ProQinase is straightforward.
- Phosphorylation shifts protein isoforms on 2D gels in the acidic direction as expected. However, the extent of the shift doesn't make sense. Mass spectrometry will be necessary to determine # of phosphates/isoforms
- More work is needed to determine the specificity of our antibodies.
- The final phosphoprotein standards should be useful in future work.

Collaborators:

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