Generation of ^{32}P-labeled MEK, ERK and VEGF-R protein standards for 2D gels

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Outline

- Protein kinase genes in the human genome
- ^{32}P labeling of ERK with MEK, a recombinant serine kinase
- ^{32}P labeling of VEGF-R, a recombinant tyrosine kinase
- Conclusions

About Protein Kinases:

- The human genome contains about 500 kinase genes:
  ~410 are serine/threonine kinases
  ~90 are tyrosine kinases
  most are not characterized

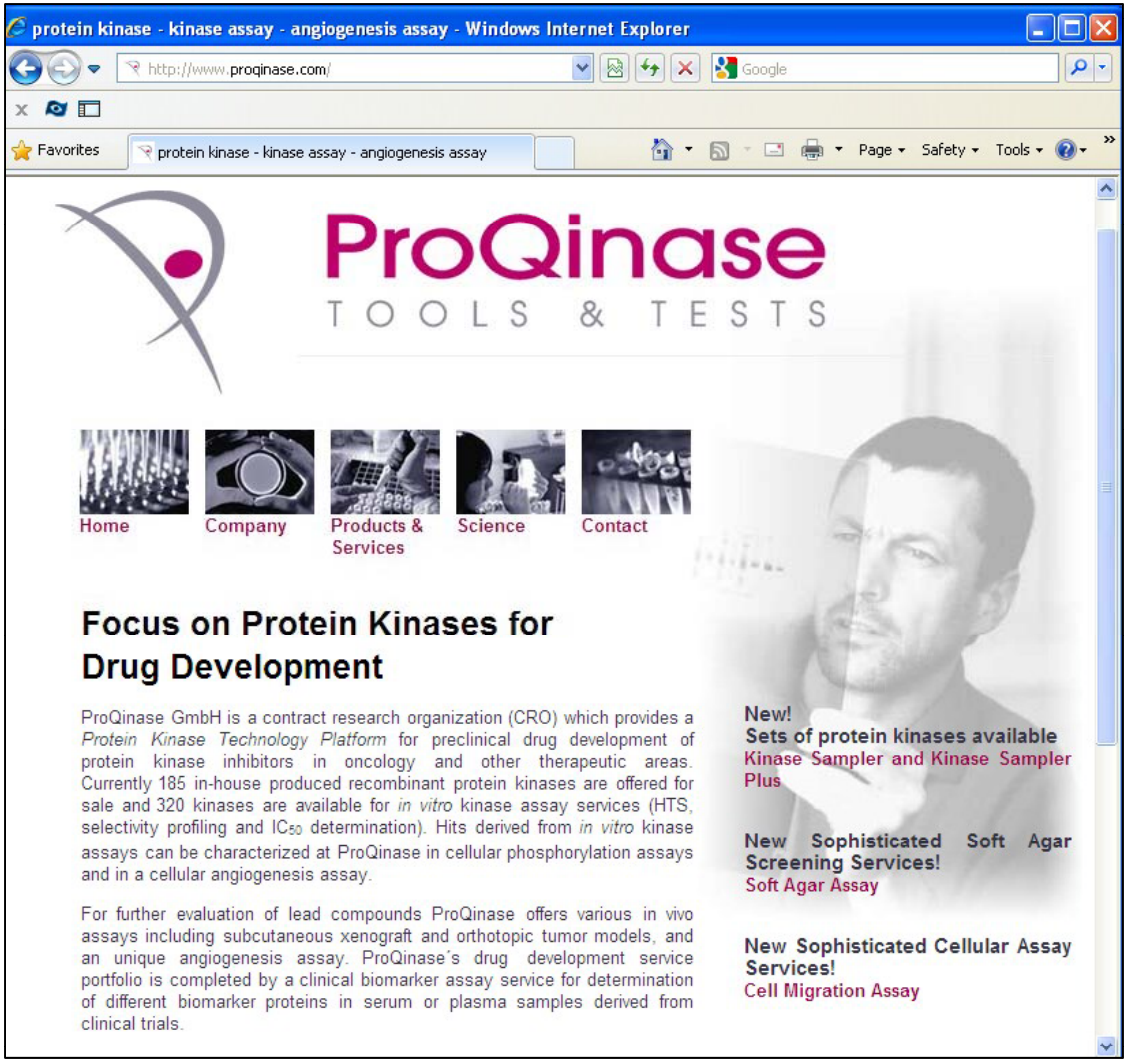
- Rough estimates suggest that 30-50% of proteins in mammalian cell lysates are serine/threonine phosphorylated. Tyrosine phosphorylation is rare and associated with mitotic events.

- Protein standards known to be phosphorylated at specific residues would be useful. But none are commercially available. (Mass spec. labs use phosphorylated *peptides*)
- So we decided to make our own phosphorylated standards using recombinant kinases and substrates.

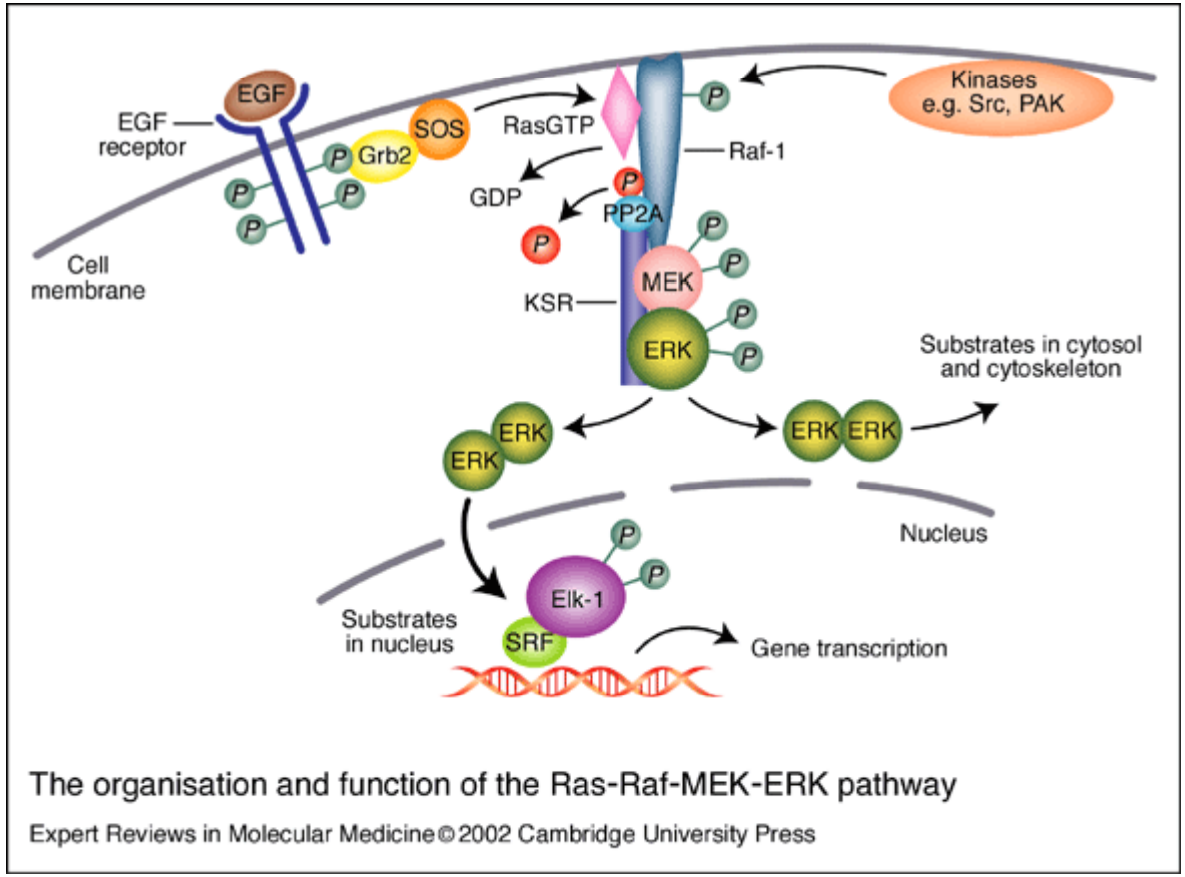
New company called ProQinase!

❖ 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.

The kinase mix was resolved by 2D Electrophoresis and Western blotting with anti-PTyr or anti-PSer/-PThr antibodies.

- A 2D gel is run, and the proteins transferred to PVDF. The membrane is stained with Coomassie blue and scanned, shaken overnight with an antibody, treated with ECL or ECL Plus and exposed to x-ray film

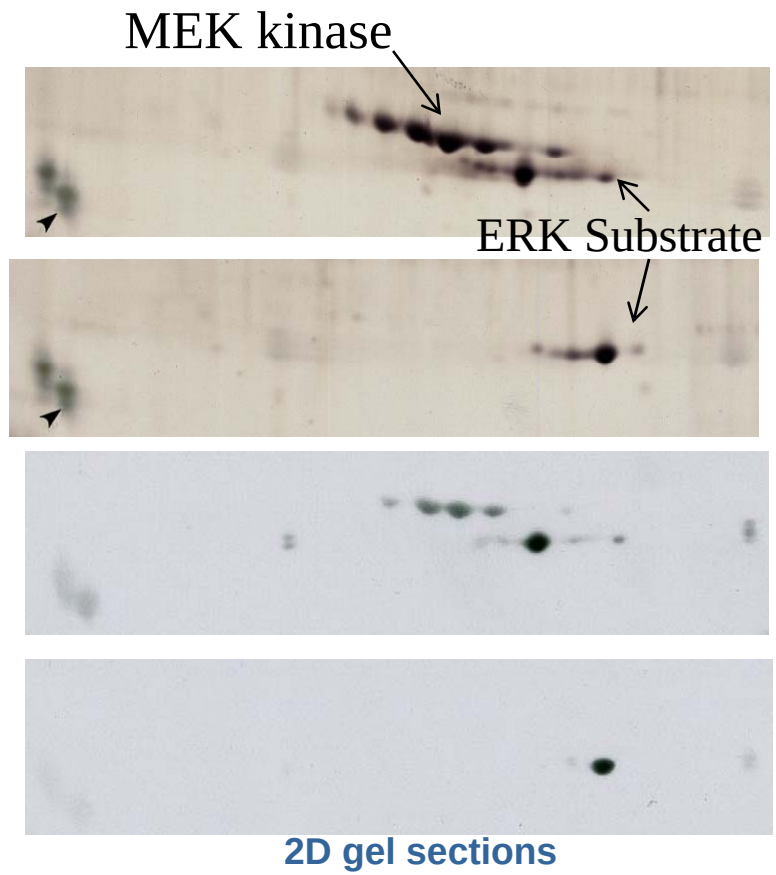
- The antibody is either:
  A generic monoclonal antibody,PY20, against phosphotyrosine or

  Qiagen monoclonal antibodies, Q5 against phosphoserine, Q7 against phosphothreonine

More method details at:
www.kendricklabs.com

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

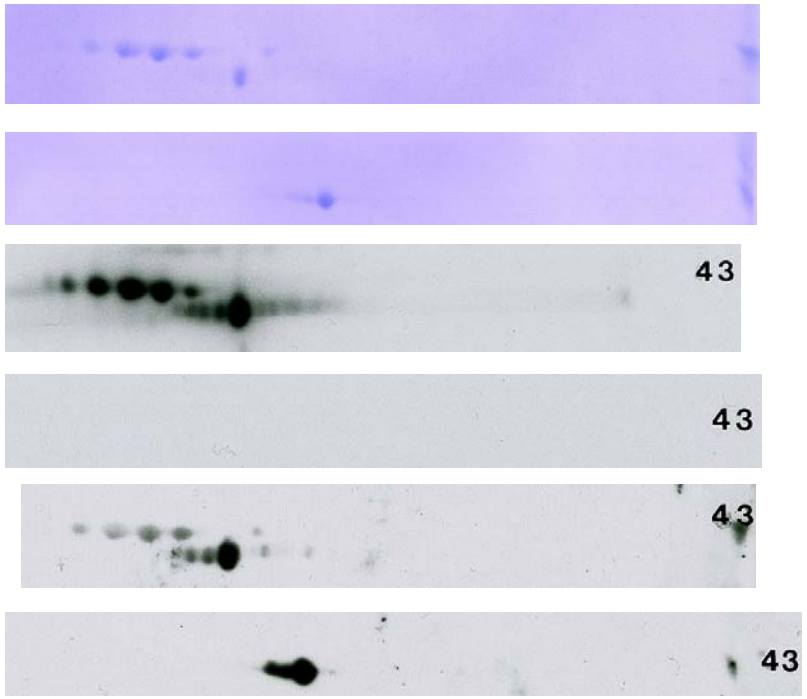


2D gel sections

The main ERK isoform moves in the acidic direction during IEF, suggesting phosphorylation is occurring when the MEK kinase is present, as expected. Surprisingly, the anti-phosphoserine ab lights up the MEK kinase as well as both forms of the Erk substrate.

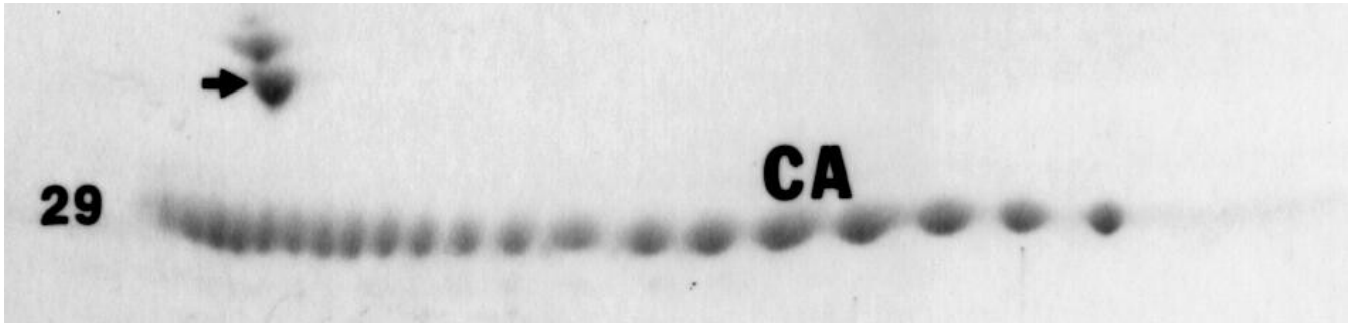
Coomassie and WB versus ^{32}P label

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 and then PSer WB was carried out. *Conclusions: The MEK kinase is clearly phosphorylated. The unphosphorylated ERK substrate lights up with the anti-phosphoserine antibody - non-specific binding, unless ERK is phosphorylated by the insect cell line.*

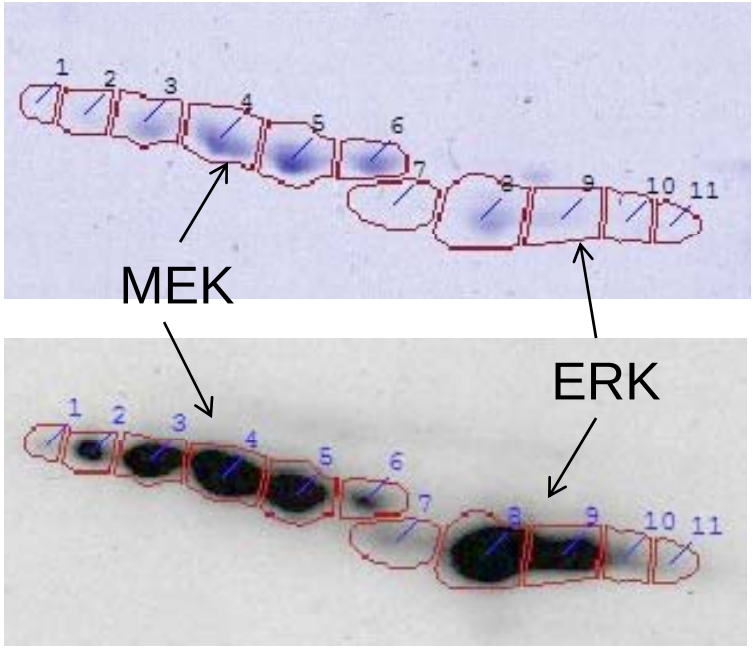
Carbamylated proteins provide evidence that 2D gels show single charge changes



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 phosphates/isoform.

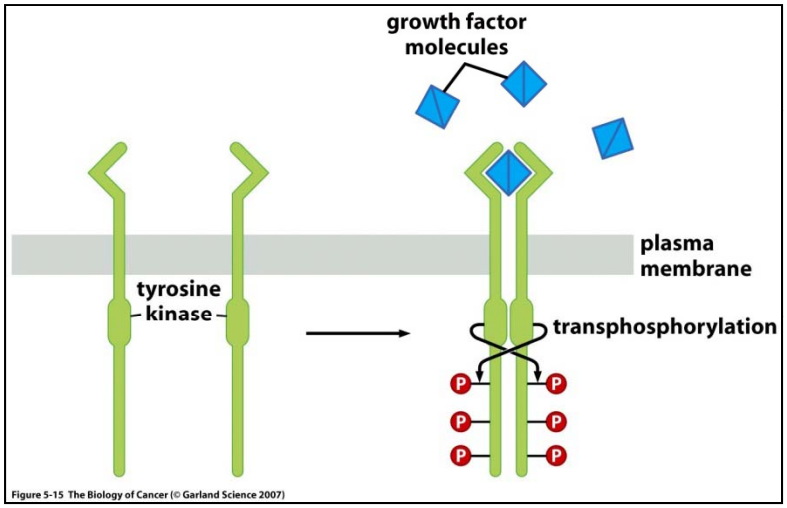
First assumption, that each aa site reaches 100% phosphorylation before the next one is filled, is naive.

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 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%

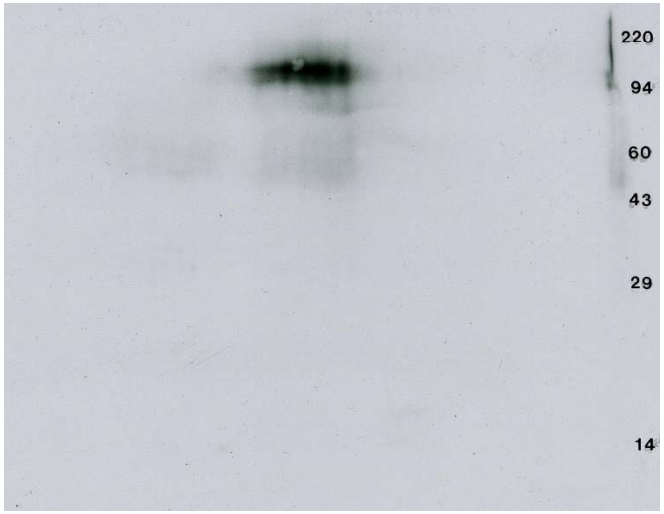
Vascular Endothelial Growth Factor Receptor (VEGF)



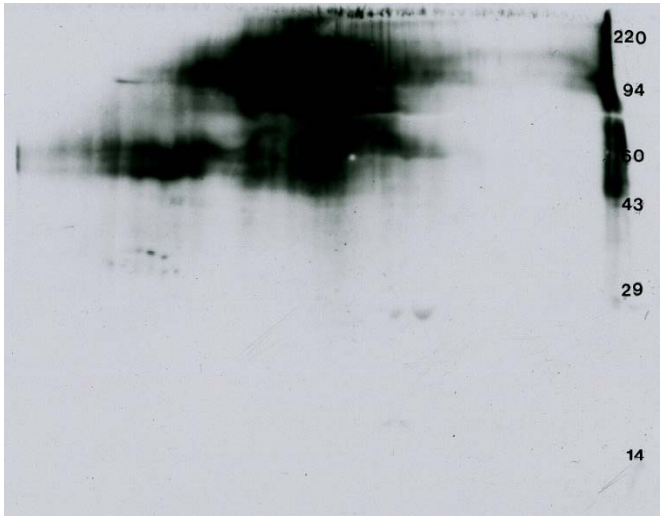
VEGF is a transmembrane receptor

- 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!



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



VEGF-R 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 seem proportional to the amount of ^{32}P incorporated. Mass spectrometry will be necessary to determine # of phosphates/isoform.
- The final standards should have many uses.