Posttranslational modification of proteins

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General classes of modification

- Biochemical event involving peptide processing
- Biochemical event stimulated by enzymes
- Chemical event driven by reactive species
- Chemical event determined by investigator

Peptide processing of polypeptides

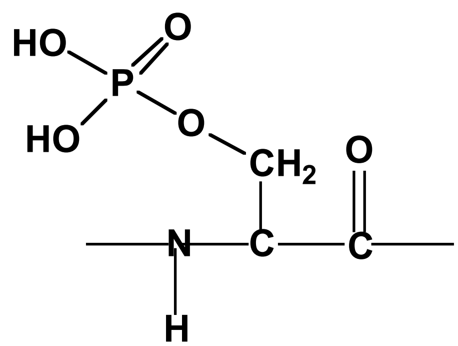
- **Head groups of membrane proteins**
- **The family of proteins in HIV that are manufactured as one protein and then hydrolyzed by a peptidase**
- **Secretogranin - a brain protein consisting of several bioactive peptides**
- **Formation of β-amyloid**

Phosphorylation of proteins

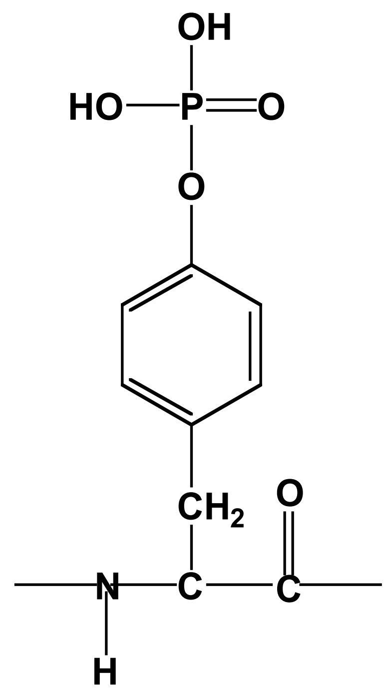
- in some cases, proteins are normally found in a stable, hyperphosphorylated state, e.g., casein

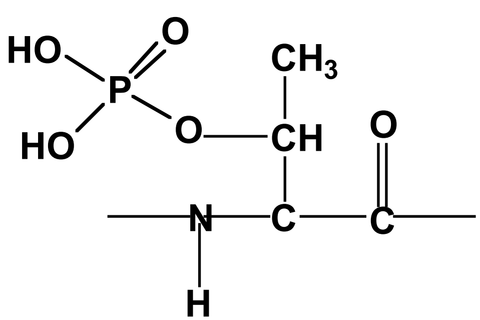
- more interestingly in many cases, it is a transient event that causes 10-100 fold increase in enzyme activity. This is the way signals are propagated through a signal transduction pathway. However, the molar abundance of phosphorylation at an individual site may only be 1-2%.

Chemistry of phosphorylation



Serine





Threonine

Tyrosine

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Mass spectrometry of proteins

- Adds H_3PO_4 (+98)
- Eliminates water (-18)
- Net change +80
- if the phosphate group is ionized (i.e., in negative ion spectra), net change is +79, otherwise it is +80 in positive ion spectra
- Can be confused with sulfate and ^{81}Br substitution

Mass spectrometry of proteins

Mass spectrometry has several advantages over other techniques

- **it is very accurate**
- **it can eliminate ambiguity by defining the site of phosphorylation**
- **it is very fast**
- **it does not require ^{32}P labeling**

<u>But it is not nearly as sensitive as ^{32}P labeling</u>

Limitations of mass spectrometry

- Although it can deliver sensitivity in the low fmol to high attomole range (similar to immunomethods), because it is a universal detection method, finding the needle in the "haystack" of all the other peptides is a challenge

- Recovering the phosphopeptides from the matrix of the sample is more important than the mass spectrometry measurement

Finding a phosphate group

Three main methods are in current use for detection of phosphopeptides

- **use of parent ion scanning**
- **affinity chromatography for enrichment of phosphopeptides**
- **phosphatase sensitivity**

Parent ion scanning to detect phosphopeptides

- **The procedure depends on the detection of the *m/z* 79 ion fragment (PO_3-) during collision-induced dissociation in a triple quadrupole instrument operating in the negative ion mode**
- **Parent ion scanning is a reversal of the more familiar daughter ion MS-MS where the parent ion is selected (in Q1) and a mass spectrum of the daughter ion fragments is obtained by scanning in Q3**
- **In parent ion scanning, the daughter ion fragment (in this case *m/z* 79) is held constant in Q3 and a mass spectrum of parent ions that give rise to the daughter ion obtained by scanning in Q1.**
- **having identified the phosphopeptides, the sample can be re-analyzed to obtain daughter ion MS-MS spectra on selected ions in the positive ion mode**

Parent ion scanning to detect phosphopeptides - Q-tof

- **Since all daughter ions are collected and sampled in this type of instrument, a parent ion scan cannot be simply obtained**
- **However, by preparing a selected ion chromatogram from all the data sets collected, individual peaks containing a phosphate fragment ion can be identified**
- **Since the MS-MS spectra are associated with this peak, spectra for the phosphorylated peptide made available**
- **This approach is enhanced if the sample is analyzed during a chromatographically effective LC-MSMS run**

Recovery and enhancement of phosphopeptides

The biggest problem in the detection of phospho-peptides is how to convert the initial sample matrix into a form suitable for mass spectrometry analysis.

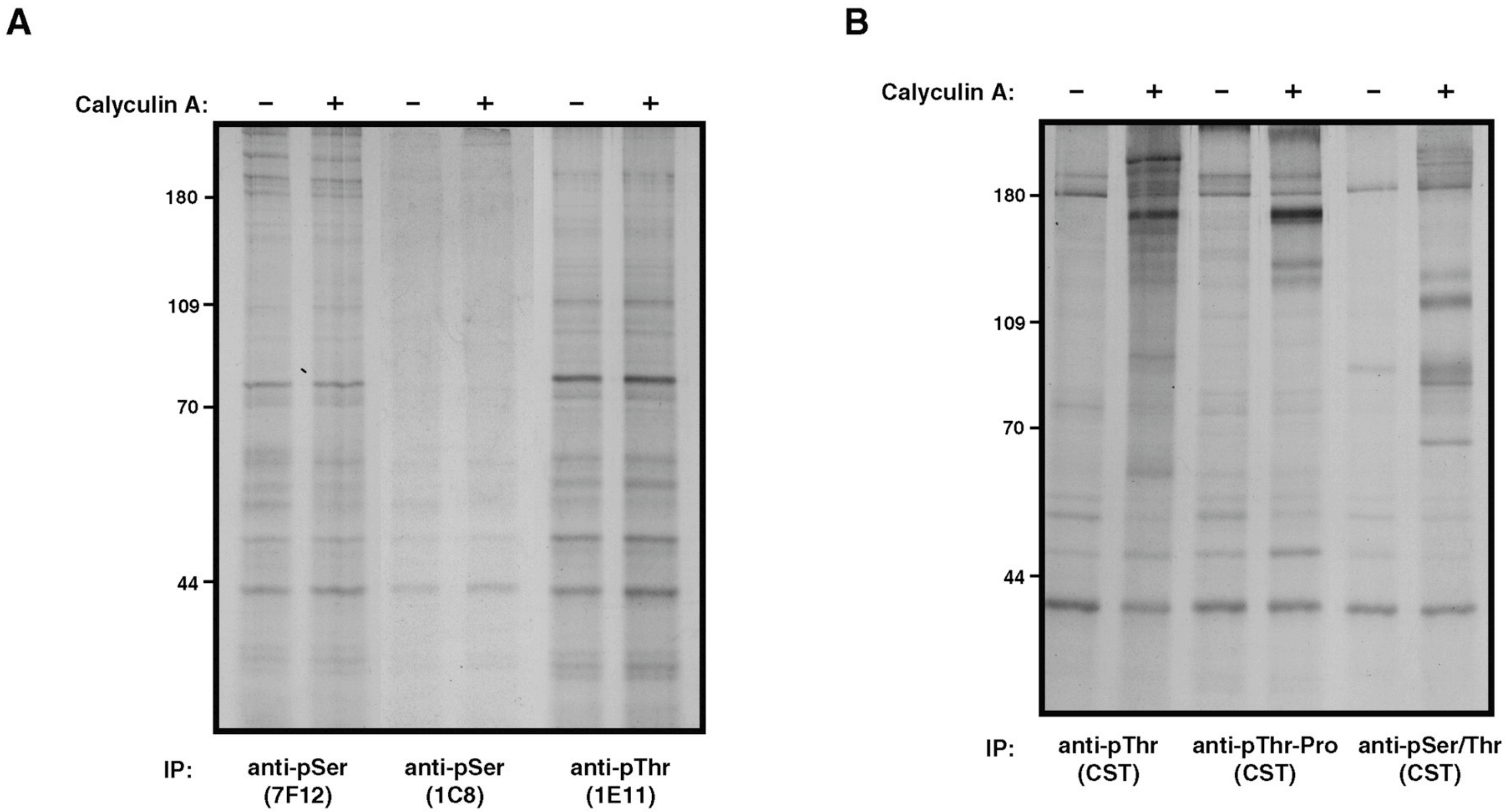
- **how to handle minute samples with minimal losses**
- **how to recover and detect all the phosphopeptides**
- **how to recover and detect the non-phosphorylated proteins to determine the extent of phosphorylation at individual sites**

Antibodies and phosphopeptides

- **In this approach, both the phosphorylated and non-phosphorylated forms of a protein may be recovered from the sample matrix**
- **This can most easily achieved by immuno-precipitation of the protein with an antibody that recognizes epitope(s) that is(are) in common with both forms**

NOTE ***that as with all immunoprecipitation methods, the best results will be achieved if the antibody is coupled to agarose beads. This allows selective immuno-absorption of the antigen, washing free of contaminating proteins, AND following elution, minimization of the amount of antibody that is in the eluate and therefore would be analyzed by mass spectrometry***

Variability of anti-phosphoserine and anti-phosphothreonine antibodies



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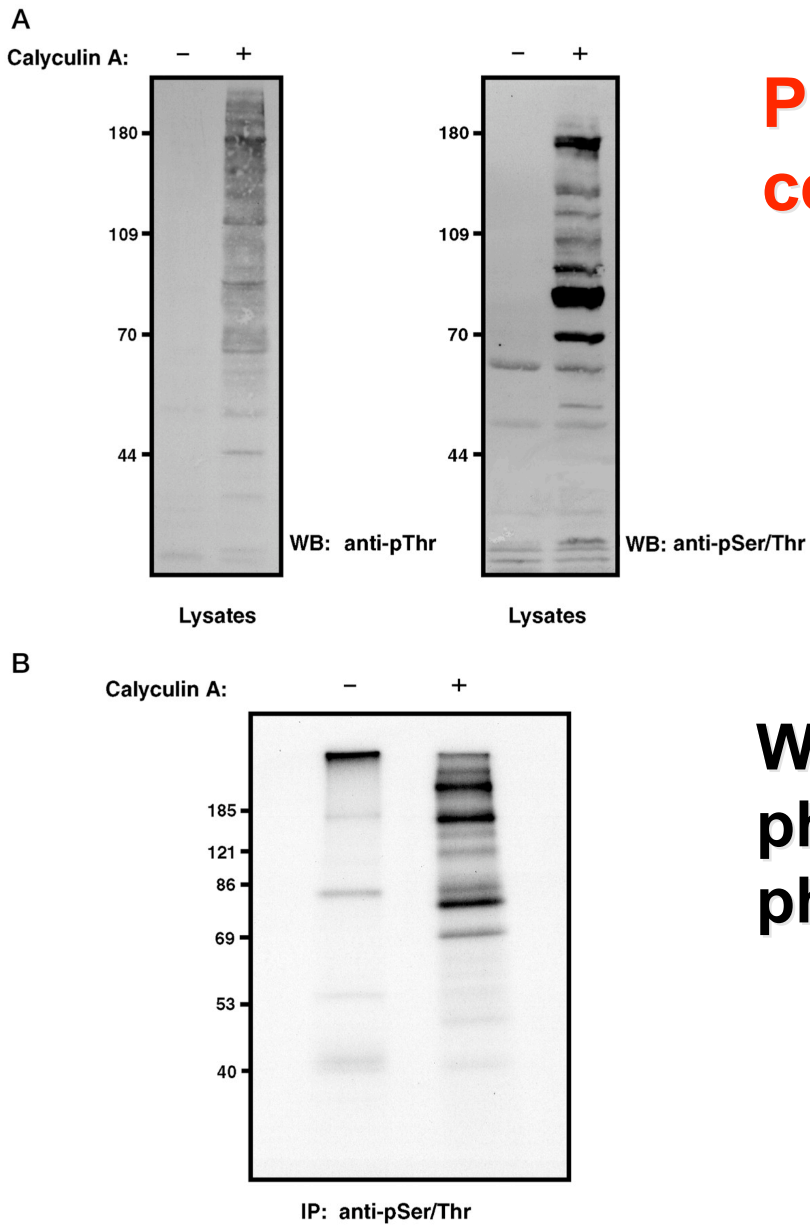
Gronborg et al., 2002



Phosphorylation in Hela cells stimulated by calyculin

Western blot analysis with anti-phosphoserine and anti-phosphotyrosine antibodies

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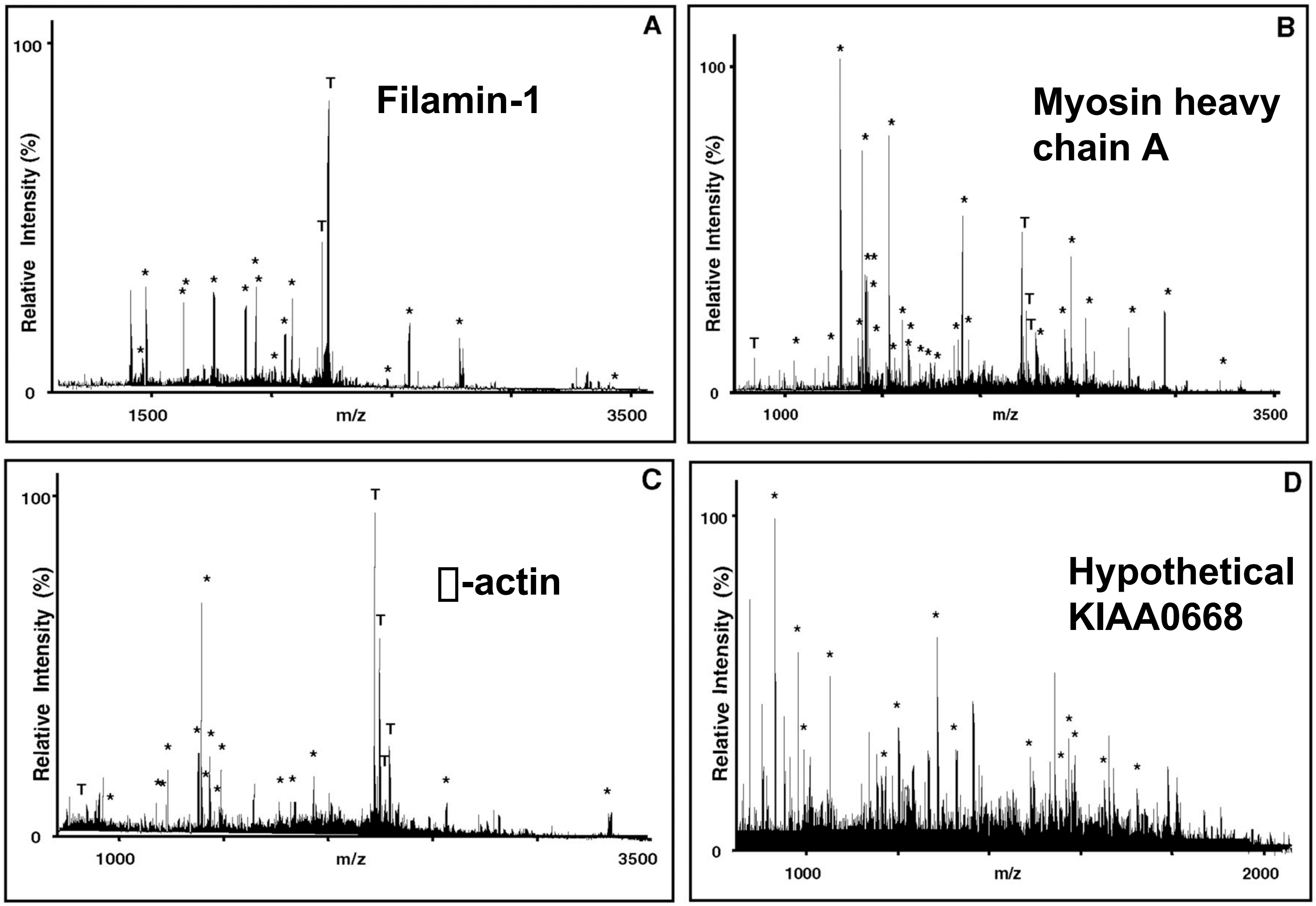
Gronborg et al., 2002

MALDI-TOF analysis of phosphorylated proteins



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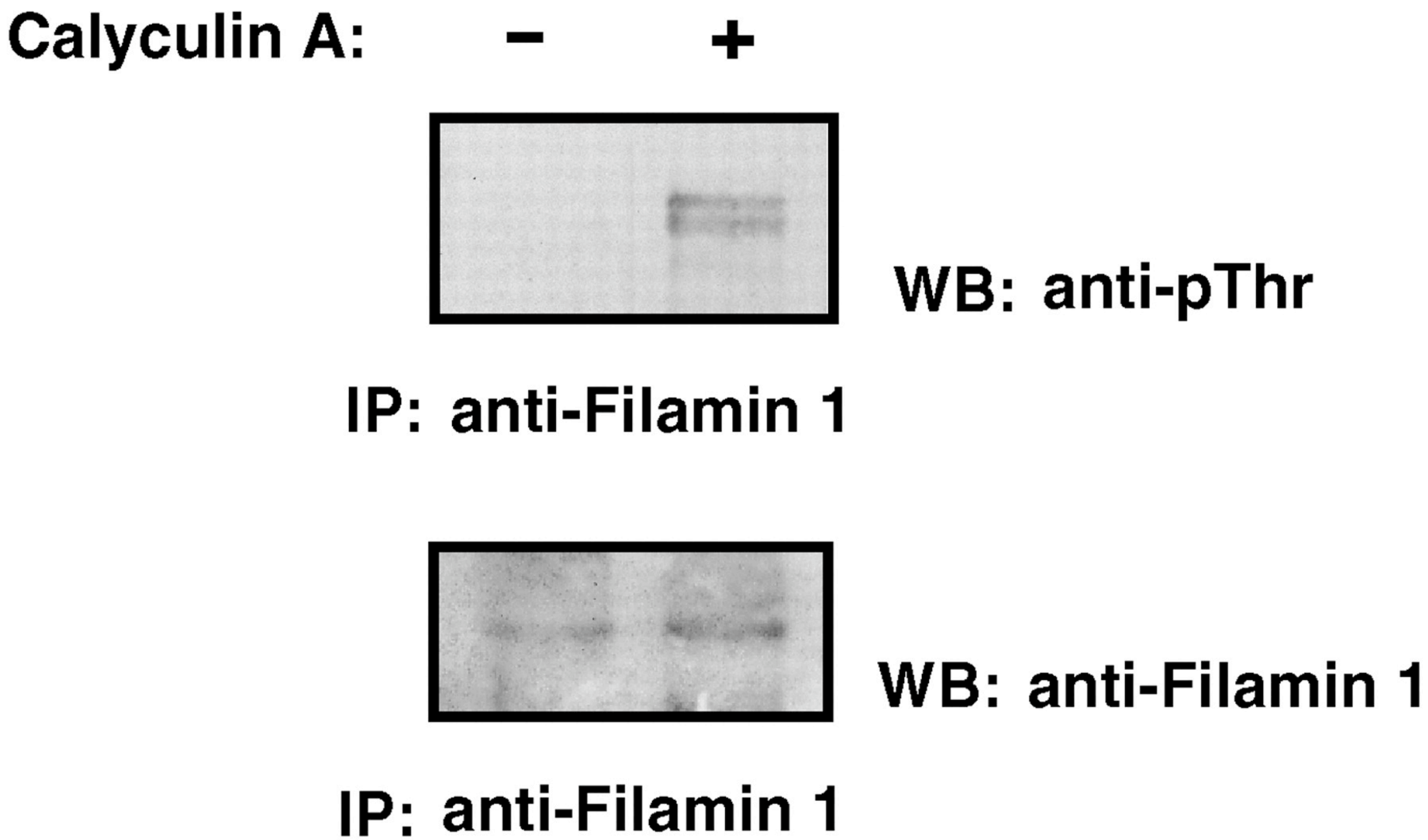
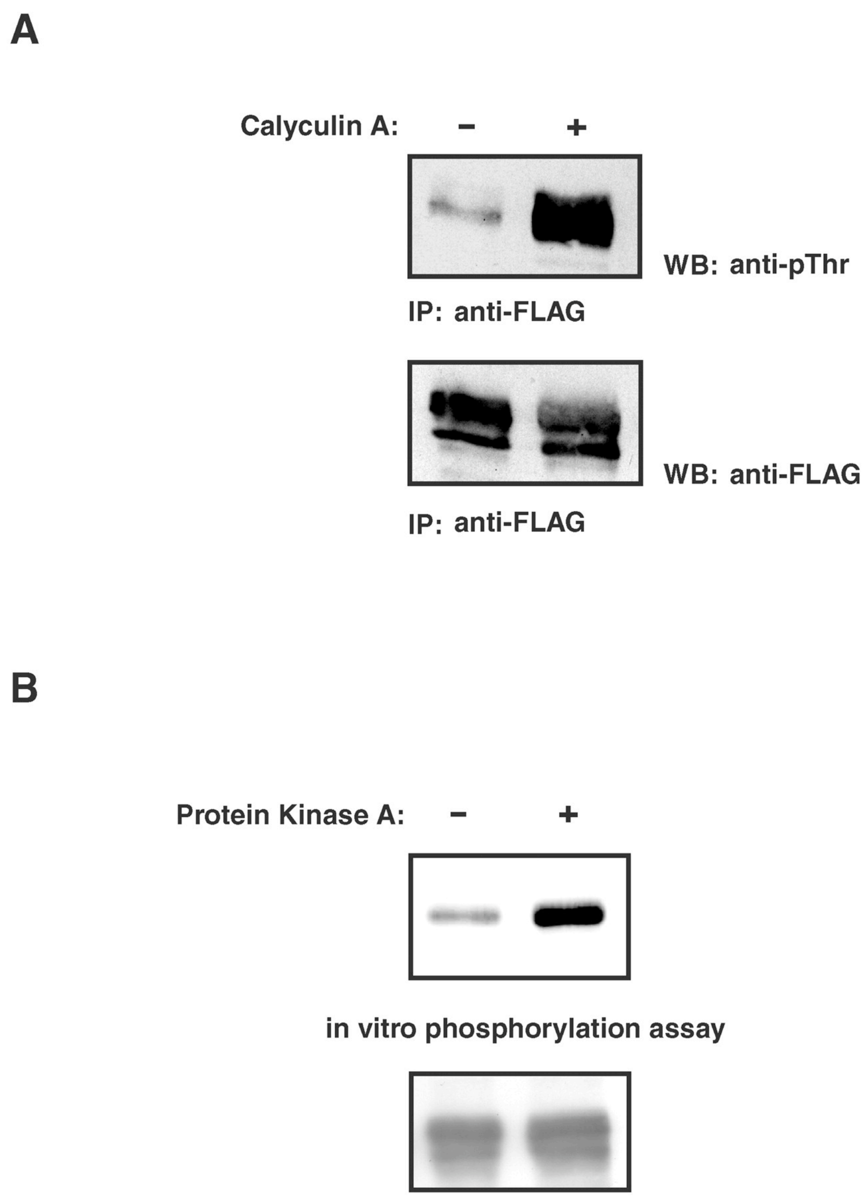
Validation of phosphorylation



Gronborg et al., 2002



KIAA0668 is Frigg

Using a FLAG epitope-tagged version of Frigg, threonine phosphorylation was demonstrated

Protein kinase A phosphorylates Frigg

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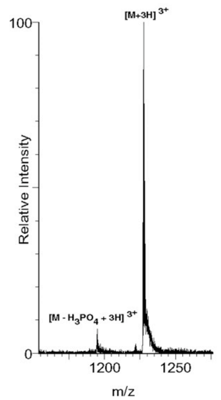
Gronborg et al., 2002

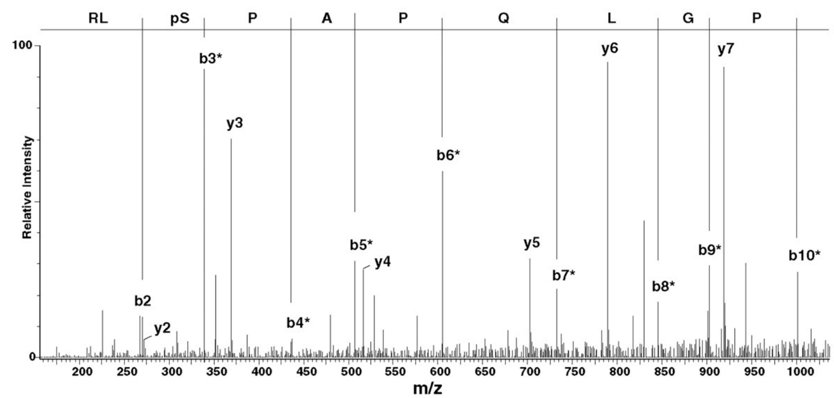
Tandem MSMS of Frigg phosphopeptides

RLpSPAPQLGPSSDAHTSYYSESLVHESWFPPR - 3+ ion

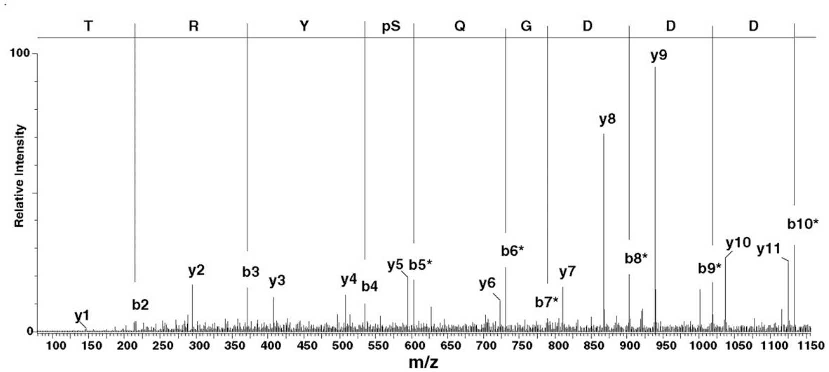
A



B



C **LTRYpSQGDDDGSSSSGGSSVAGSQSTLFK - 4+ ion**



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Gronborg et al., 2002

Detection of phosphopeptides based on their sensitivity to phosphatase

- **An alternative source of potentially phosphorylated proteins are individual spots on 2D-IEF/SDS gels. The protein preparation so isolated is either hydrolyzed by trypsin in solution (or in the gel piece) or using solid-phase trypsin**

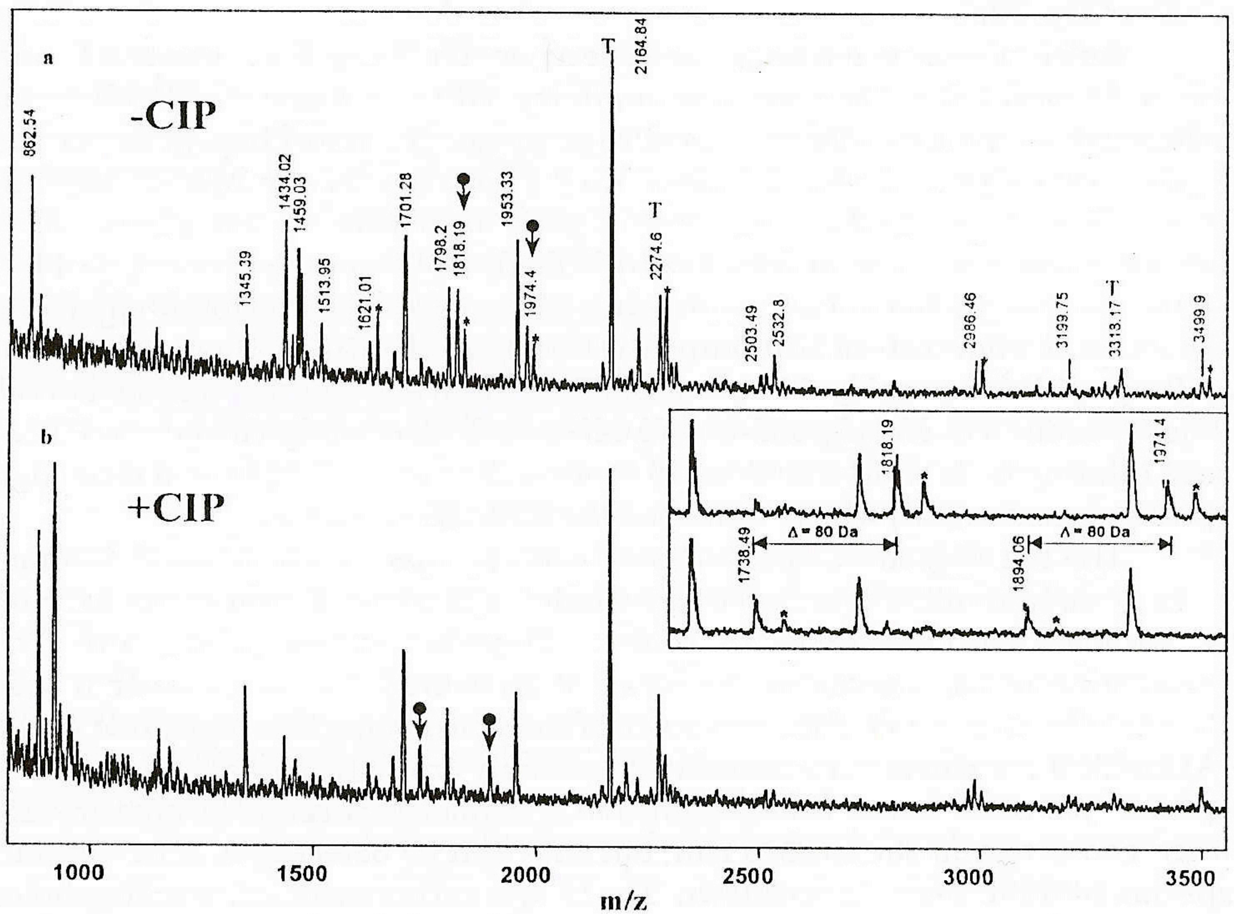
- **One portion of the resulting tryptic peptides (in 50% acetonitrile:water) is analyzed by MALDI-TOF-MS. A second portion is diluted into 50 mM NH_4HCO_3 buffer and reacted with 0.5 U calf intestinal alkaline phosphatase at 37^{o}C for 30 min. Sample is dried with a SpeedVac, redissolved in 50% acetonitrile:water, and re-analyzed by MALDI-TOF-MS**

Identifying phosphopeptides using alkaline phosphatase

- Phosphopeptides shift down by m/z 80 (or units of 80 in the case of multiply phosphorylated peptides)

- The peaks identified as phosphopeptides can then be analyzed in a nanoelectrospray experiment where collision-induced dissociation is used to determine the identity of the peptide and the phosphorylation site in the sequence

Detecting a phosphopeptide with alkaline phosphatase



Zhang et al., 2000
MS in Biology & Medicine

Selective enhancement of phospho-peptides in tryptic digests

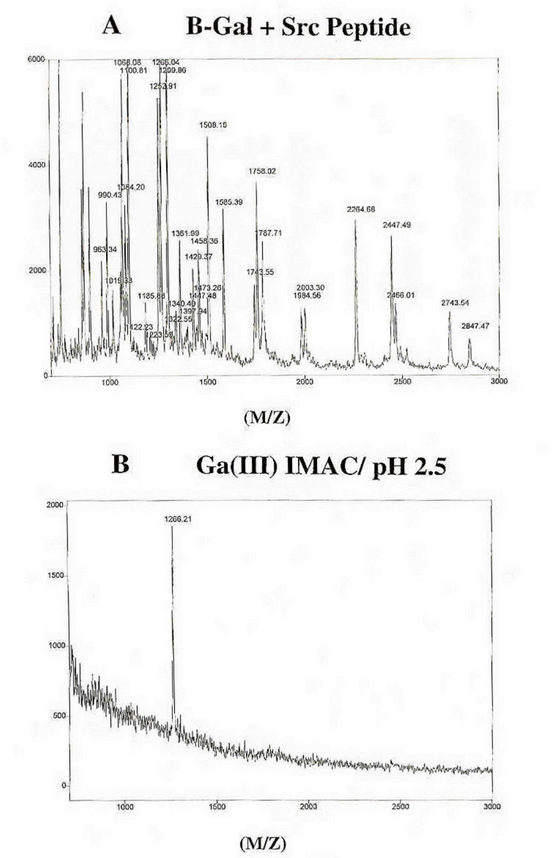
- Immobilized metal affinity chromatography (IMAC). Similar to Ni-affinity resins used in the purification of 6xHis-tagged proteins. The affinity phase can be charged with different metal ions (as their chlorides)
- Fe(III) and Ga(III), and to a lesser extent Zr(IV), were the most effective for the recovery of two synthetic phosphopeptides
- A tryptic digest containing both phosphorylated and non-phosphorylated peptides is passed over the IMAC column at acid pH (pH 2.5-3)
- The column is washed with 0.1 M acetic acid to remove unbound peptides

Selective enhancement of phospho-peptides in tryptic digests

An alternative elution method is to use ammonium hydroxide (0.075%) which is volatile and the salt it forms will be with acetic acid - this does not interfere with the electrospray ionization process

- **however, elution from Fe(III) and Zr(IV) matrices was poor - in this application Ga(III)-IMAC is a far superior phase**

Selective recovery of phosphopeptide using a Ga^{3+}-IMAC column



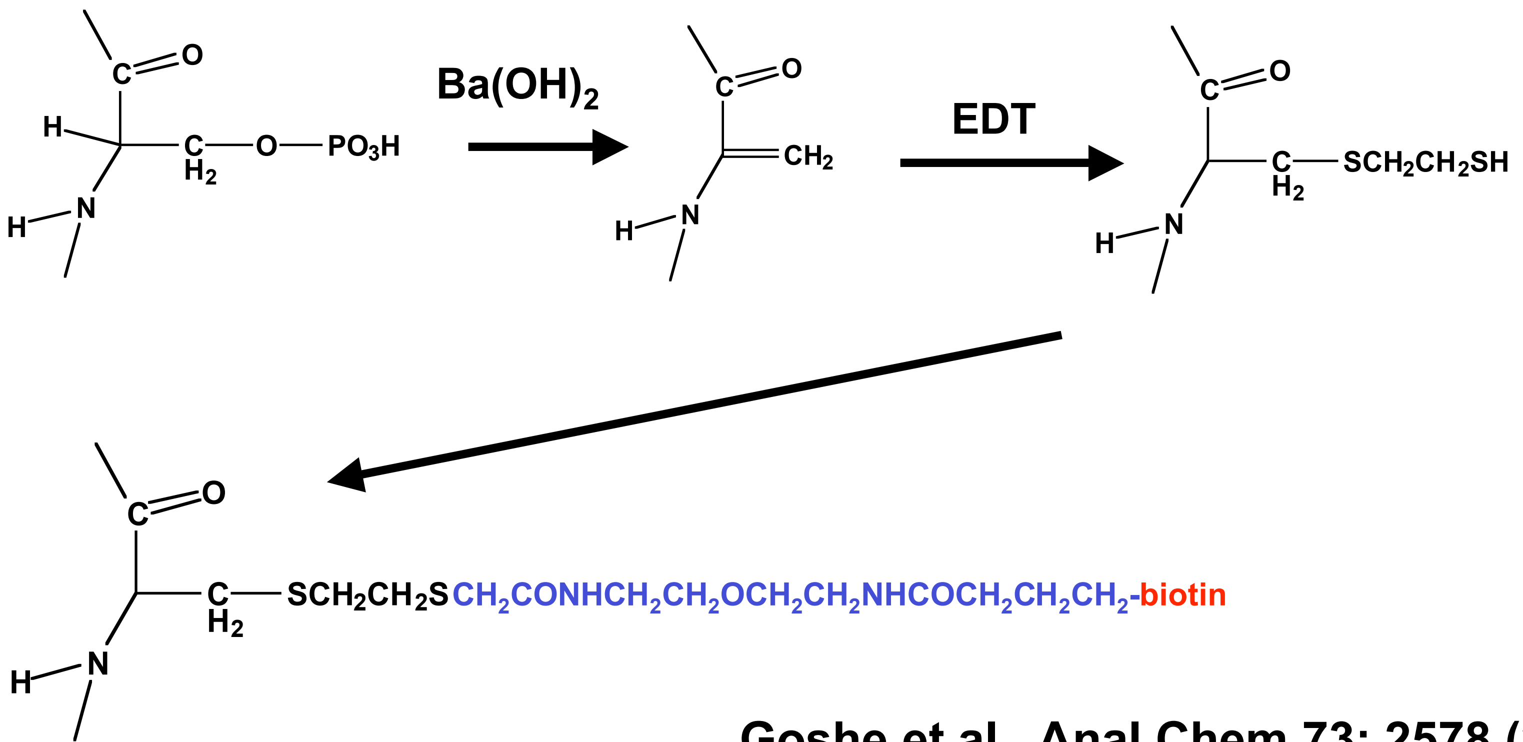
A phosphorylated peptide from Src was mixed with a tryptic digest of β-Gal

The upper panel shows the total MALDI

The lower one is the peptide retained by the Ga^{3+}-IMAC column

Tempst et al., 2000

Selective biotinylation of phospho-groups



Goshe et al., Anal Chem 73: 2578 (2001)
Adamcyk et al., Rapid Commun Mass Spec 15; 1481 (2001)

How to identify phosphorylated peaks by searching databases

- **The databases you have already seen have some ability to predict the expected masses for a limited number of posttranslational modifications**

  - **MASCOT (http://www.matrixscience.com)**
  - **PROTEIN PROSPECTOR (http://prospector.ucsf.edu/)**
  - **PROFOUND (http://www.proteometrics.com/prowl.cgi/ProFound.exe)**

How to identify posttranslational modifications at a new site

FindMod at

http://www.expasy.ch/tools/findmod/

It examines mass fingerprinting data for mass differences between empirical and theoretical peptides. If the mass difference corresponds to a known modification, it also makes intelligent guesses as to the site of modification.

FindMod

Modifications considered are:

acetylation	amidation	biotinylation
C-mannosylation	deamidation	flavinylation
farnesylation	formylation	geranyl-geranylation
g-carboxyglutamic acid	hydroxylation	lipoylation
methylation	myristoylation	N-acyl diglyceride
O-GlcNac	palmitoylation	phosphorylation
pyridoxal phosphate	phospho-pantetheine	pyrrolidone-carboxylic acid
sulfation		

NOTE that none of the common chemical modifications (alkylation of sulfhydryl groups with iodoacetic acid, iodoacetamide, 4-vinylpyridine, and acrylamide) were included.

The list also omits nitration and the recently discovered halogenation of peptides.

See the article by Wilkins et al. (1999) in J. Mol Biol. for details on FindMod

Site for compilation of PTMs

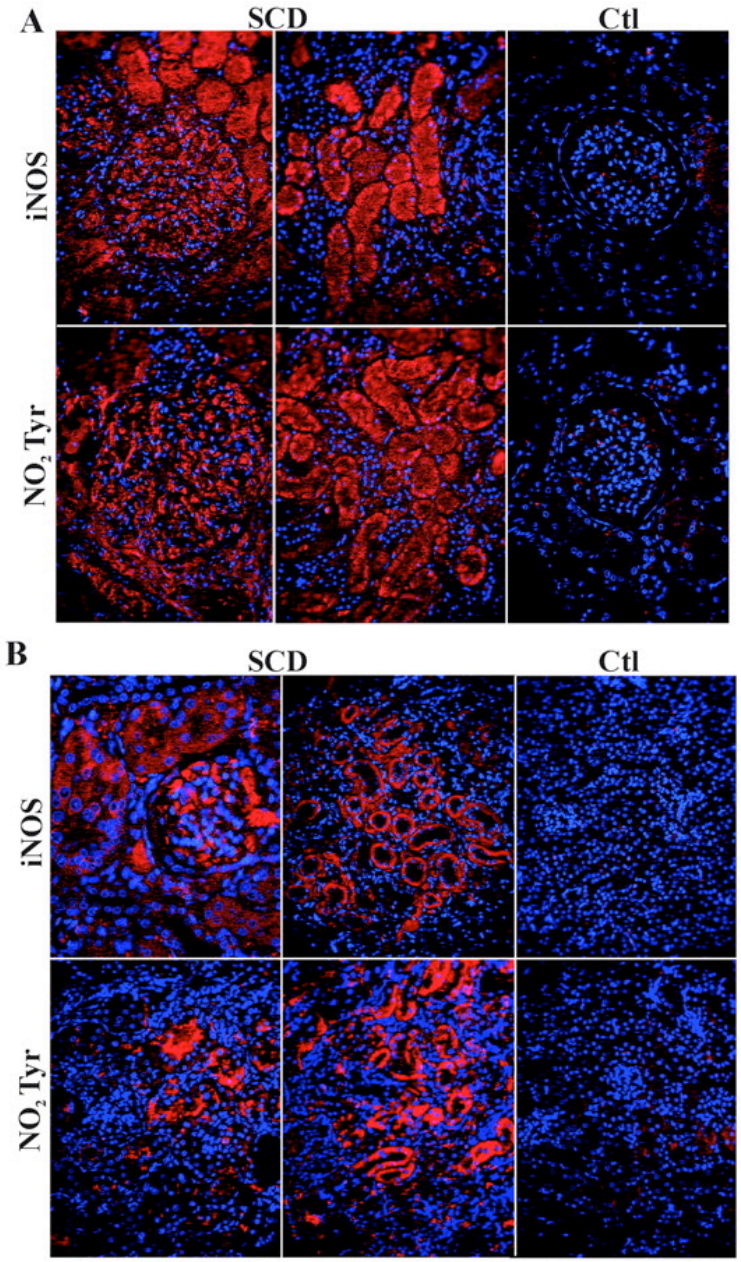
https://www.abrf.org/index.cfm/dm.home

This site was put together by Ken Mitchelhill, Len Packman and friends

Currently ranges from dephospho (-79) to (Hex)3-HexNAc-(dHex)HexNAc (+1,039)

Nitration of proteins

- Peroxynitrite is a highly oxidizing and nitrating specie produced by the reaction of nitric oxide and superoxide
- UAB has an important place in the identification of nitrated proteins
  - 1996 Greis et al., Arch Biochem Biophys 335:396 (Surfactant protein A)
  - 1997 Crow et al., J Neurochem 69:1945 (neurofilament-L)
  - 2000 Cassina et al., J. Biol Chem 275:21409 (cytochrome C)
  - 2003 Aslan et al., J Biol Chem 278:4194 (actin)



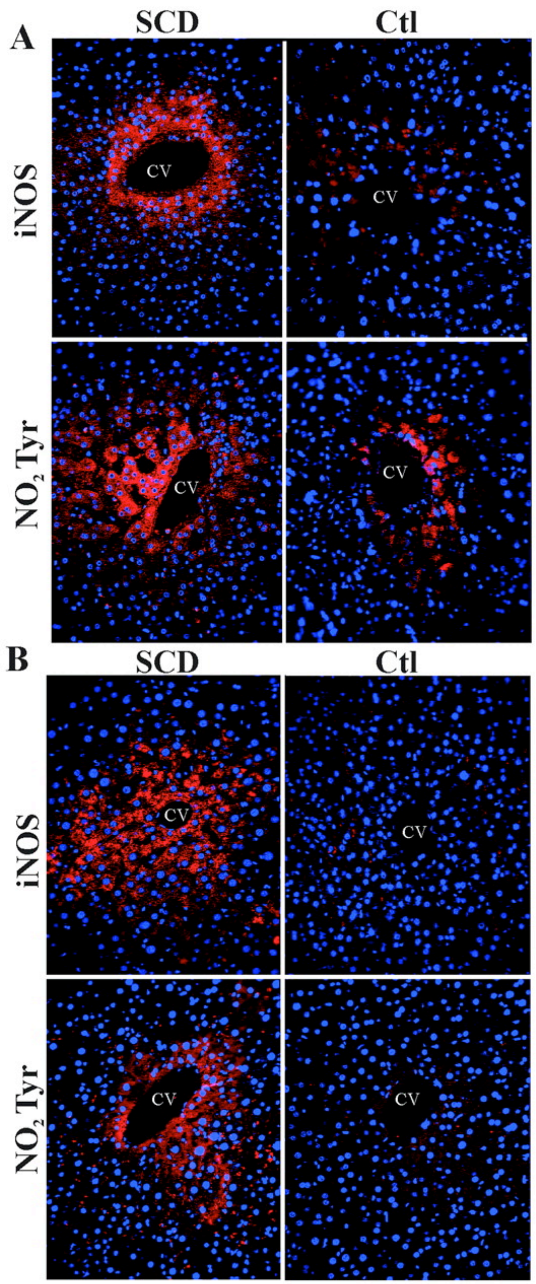
Formation of nitrotyrosine in the kidney is a consequence of sickle cell anemia, a disease due to a point mutation in the hemoglobin gene

In this slide, there is intense immunoreactivity with iNOS, an enzyme that generates nitric oxide (NO) in the glomeruli and the proximal and distal tubules. Similarly, proteins containing 3-nitrotyrosine light up in glomeruli and the tubules.

A = human kidney

B = kidney from transgenic mouse with the hemoglobin gene mutation

Aslan et al., JBC 278:4194



Immunohistochemical analysis reveals that nitration is related to vascular events - the increase in iNOS and NO_2Tyr occurs around the central vein in the hepatocyte

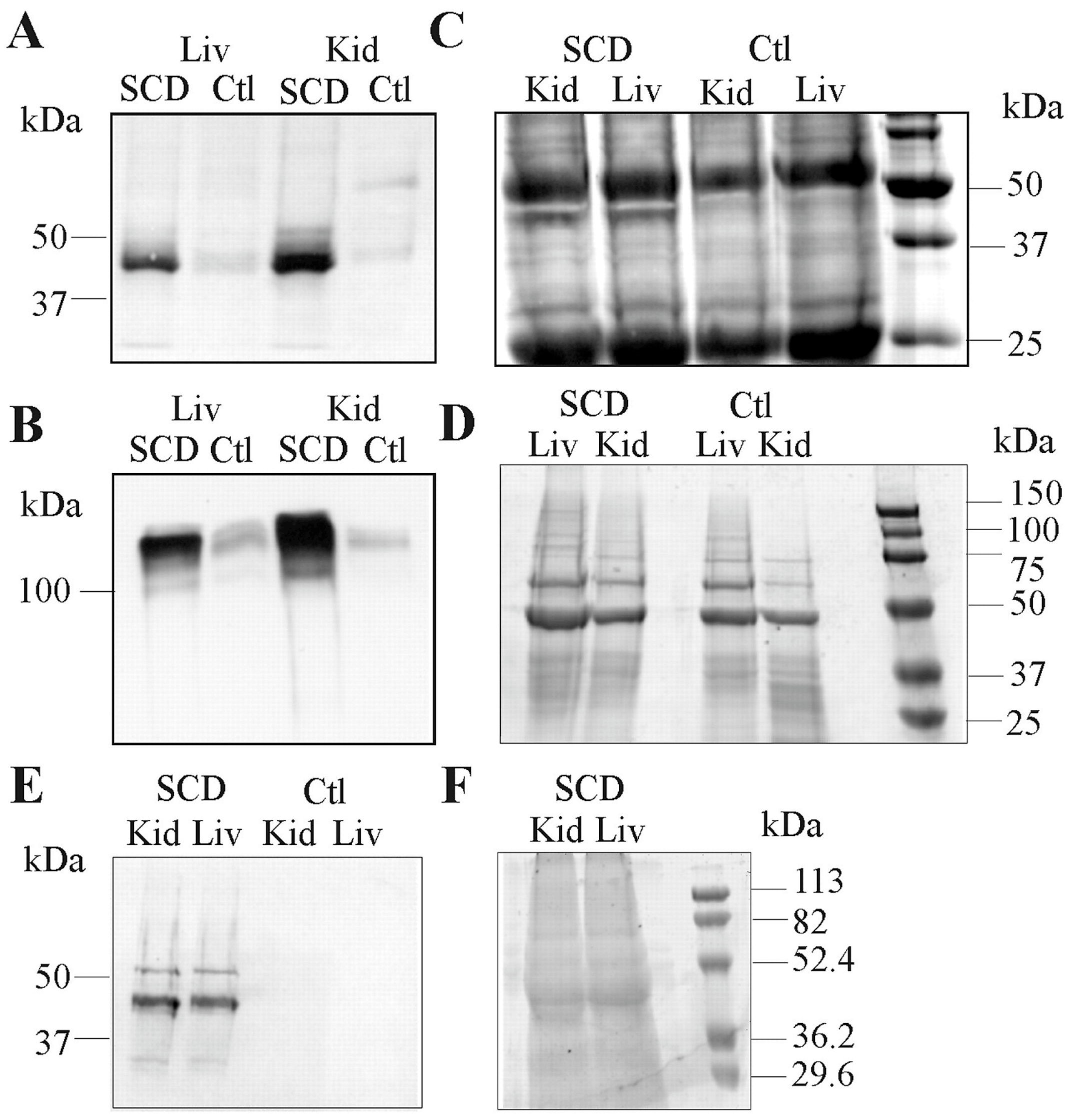
$$NO\cdot + O_2^{-\cdot} = ONO_2^{-}$$

NO reacts with another radical, superoxide anion (produced by mitochondria) to form peroxynitrite, the chemical that causes nitration

A = human kidney

B = kidney from transgenic mouse with the hemoglobin gene mutation

Aslan et al., JBC 278:4194



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SDS-PAGE and Western blot analysis

A = anti-nitrotyrosine and liver and kidney homogenates

B = anti-iNOS

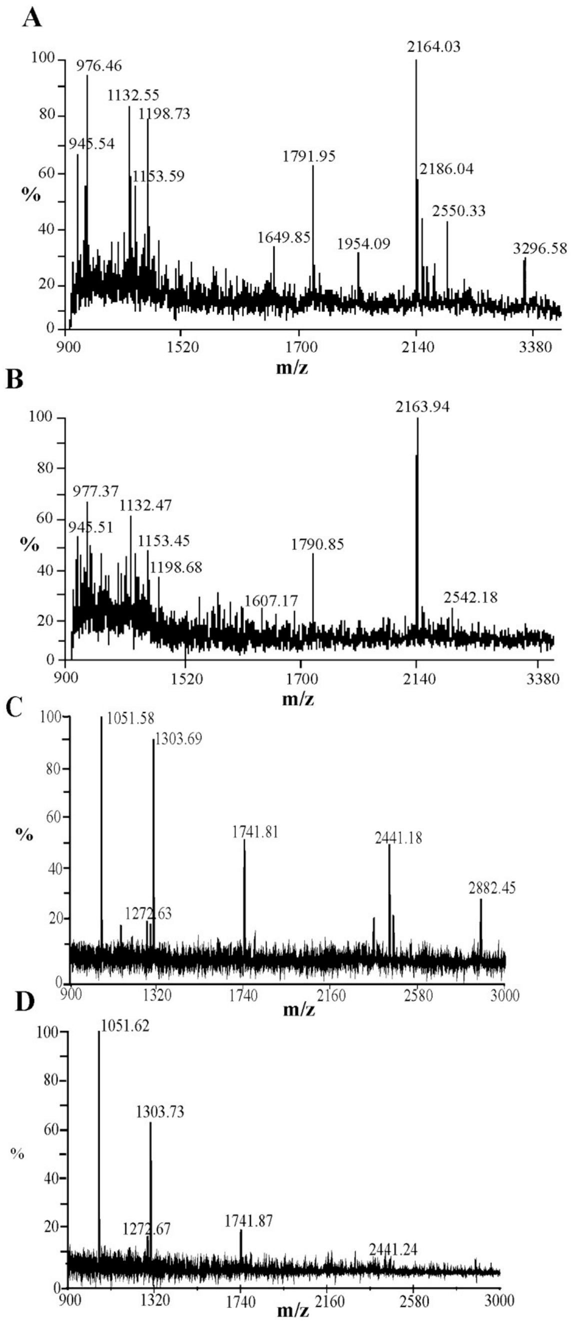
C = immunoprecitated NO_2Tyr proteins run on SDS-PAGE and stained by Coomassie Blue

D = actin-enriched proteins run on SDS-PAGE and stained with Coomassie Blue

E = Western blot with anti-nitrotyrosine of actin-enriched proteins

F = Actin-enriched proteins bound to anti-nitrotyrosine affinity phase, eluted and run on SDS-PAGE and stained with Coomassie Blue

Aslan et al., JBC 278:4194



MALDI-TOF analysis of proteins from mouse liver and kidney immunoprecipitated with NO_2Tyr antibody

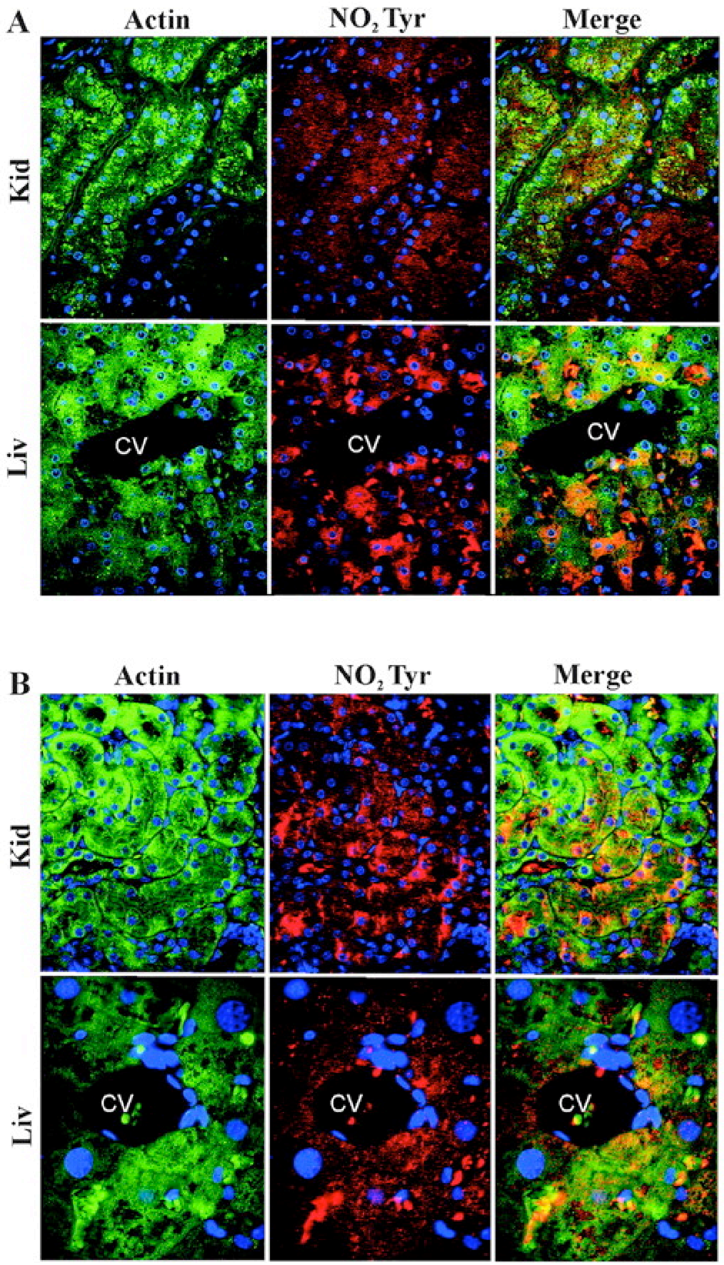
A = 42 kDa protein from mouse liver - actin

B = 42 kDa protein from mouse kidney - actin

C = 53 kDa protein from mouse liver - vitamin D-binding protein

D = 53 kDa protein from mouse kidney - vitamin D-binding protein

Steve Barnes 2-11-03 Aslan et al., JBC 278:4194



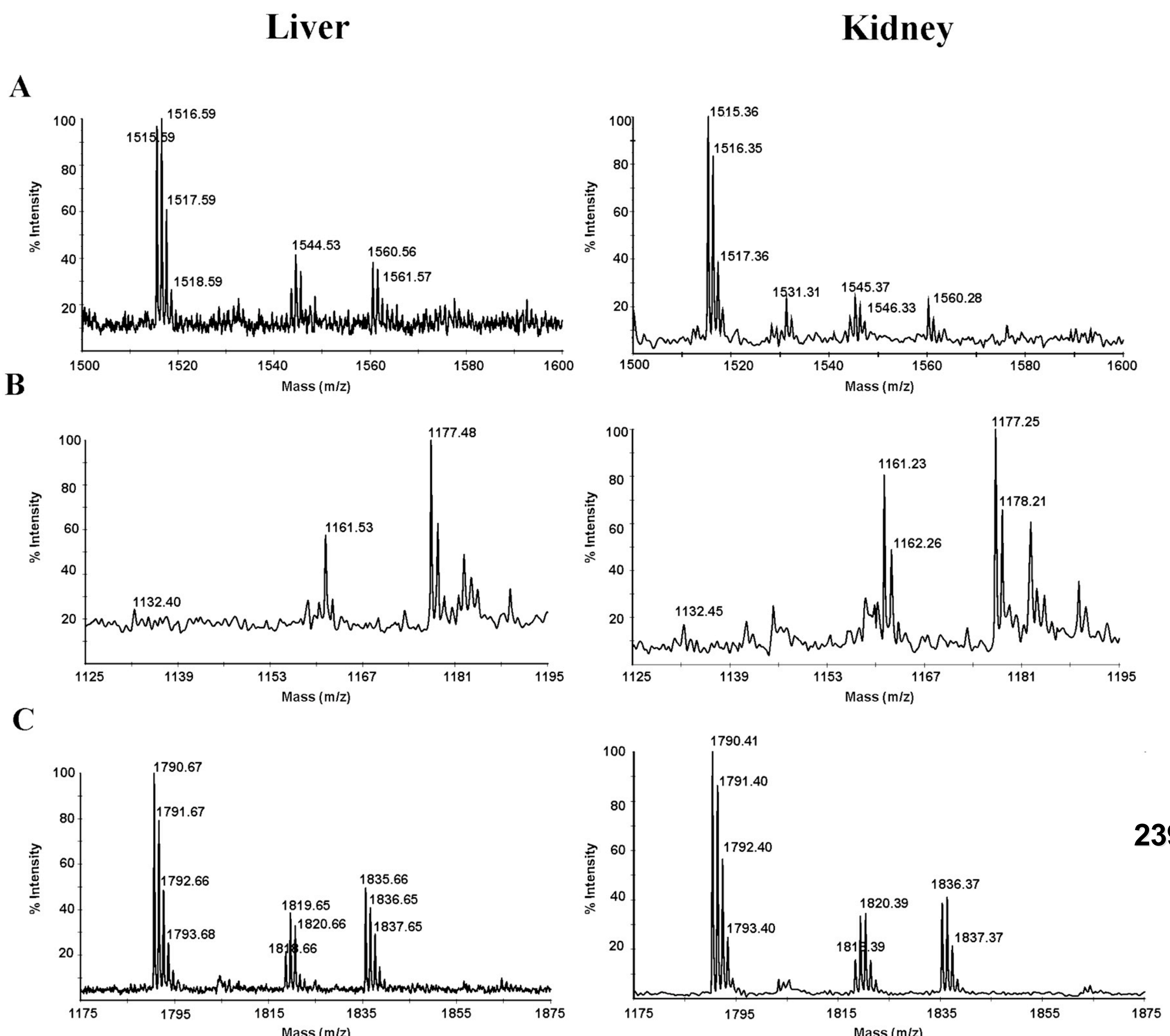
Evidence that actin and the anti-NO_2Tyr immunoreactivity are co-localized

Tissue sections of kidney and liver from humans (A) and mice (B) with sickle cell anemia

Actin is labeled in green and NO_2Tyr in red. The orange colored regions are the sites of co-localization of actin and nitrotyrosine

Aslan et al., JBC 278:4194

MALDI-TOF identification of NO_2Tyr peptides in actin



^{85}IWHHTFYNELR^{95}

^{197}GYSFTTTAER^{206}

^{239}SYELPDGQVITIGNER^{254}

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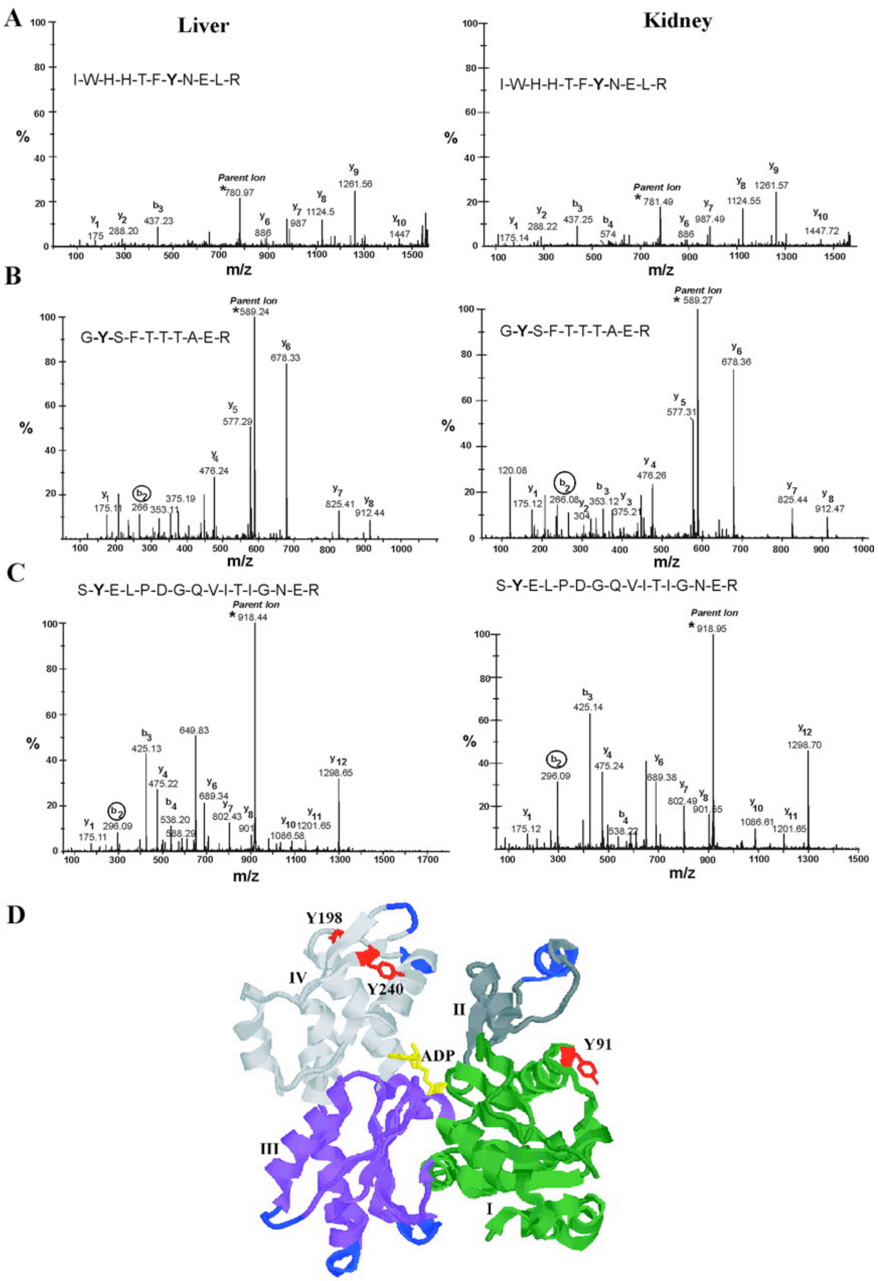
Confirmation of nitrated actin peptide identities by tandem MS-MS from in vivo experiments



b_2 ion $= [57+1+163+45]$
$= 266$

b_2 ion $= [87+1+163+45]$
$= 296$

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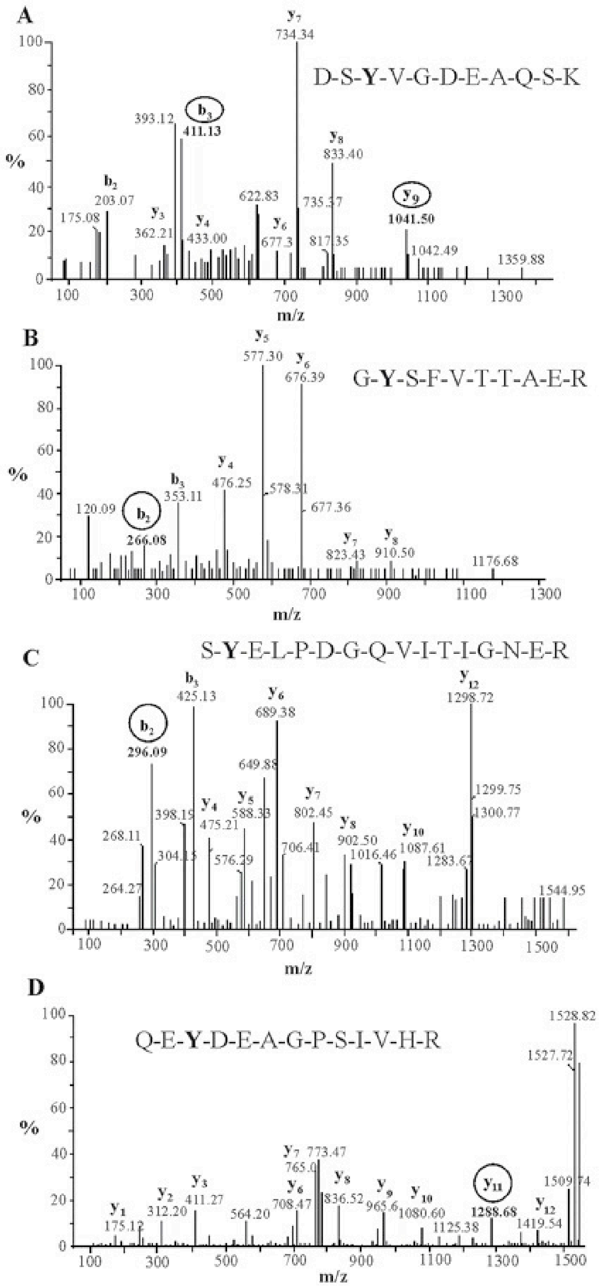
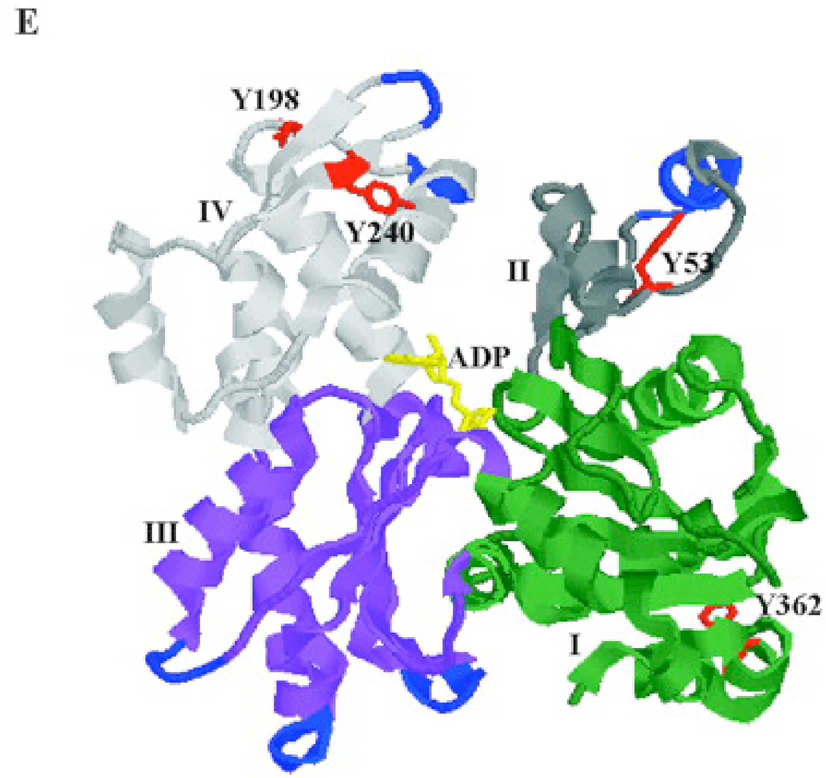
Aslan et al., JBC 278:4194

Nitrated peptides formed in *in vitro* experiments with peroxynitrite parallel those found *in vivo*





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Aslan et al., JBC 278:4194

Key points to remember

- Actin is a highly abundant protein in cells
- Proteins in the 40-44 kDa range are frequently heavily contaminated with actin
- 2D-IEF/SDS-PAGE can help separate actin from other proteins

- Actin can be nitrated
- However, nitration is a low abundance event
- So, even detection of nitration of actin requires a preliminary immunopurification