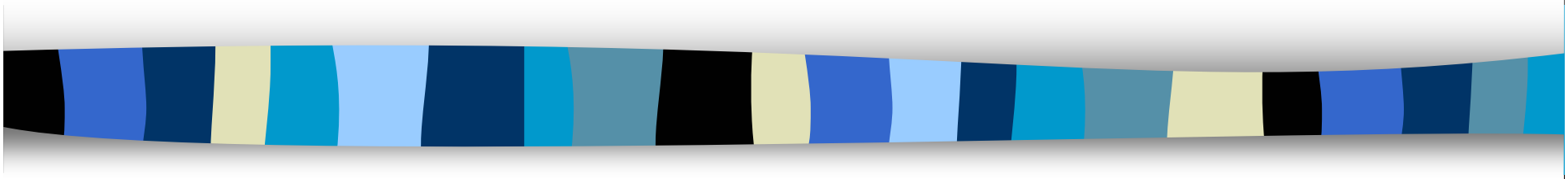
Aplikovaná chemie a biochemie



Přednáška č. 1

Úvod

Stručný přehled:

- detekce exprese, lokalizace, syntézy, degradace a funkcí proteinů; protilátky;
- detekce exprese na úrovni RNA; využití siRNA pro manipulace s expresí genů; microarrays;
- práce s DNA; využití vektorů pro transfekci živočišných buněk *in vitro*; aktivace transkripce;
- lipidy a polysacharidy – izolace, separace a identifikace;
- nízkomolekulární látky – separační a analytické techniky, HPLC techniky a modifikace;
- využití chemických inhibitorů,
- modelové organismy; transgenní zvířata.

Náplň předmětu:

- přednášky;
- seminář;

Literatura:

- Molecular Biology of The Cell (s.469-580), http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=books

- Principles and Techniques of Practical Biochemistry

http://assets.cambridge.org/97805216/58737/toc/9780521658737_toc.pdf

Primární literatura

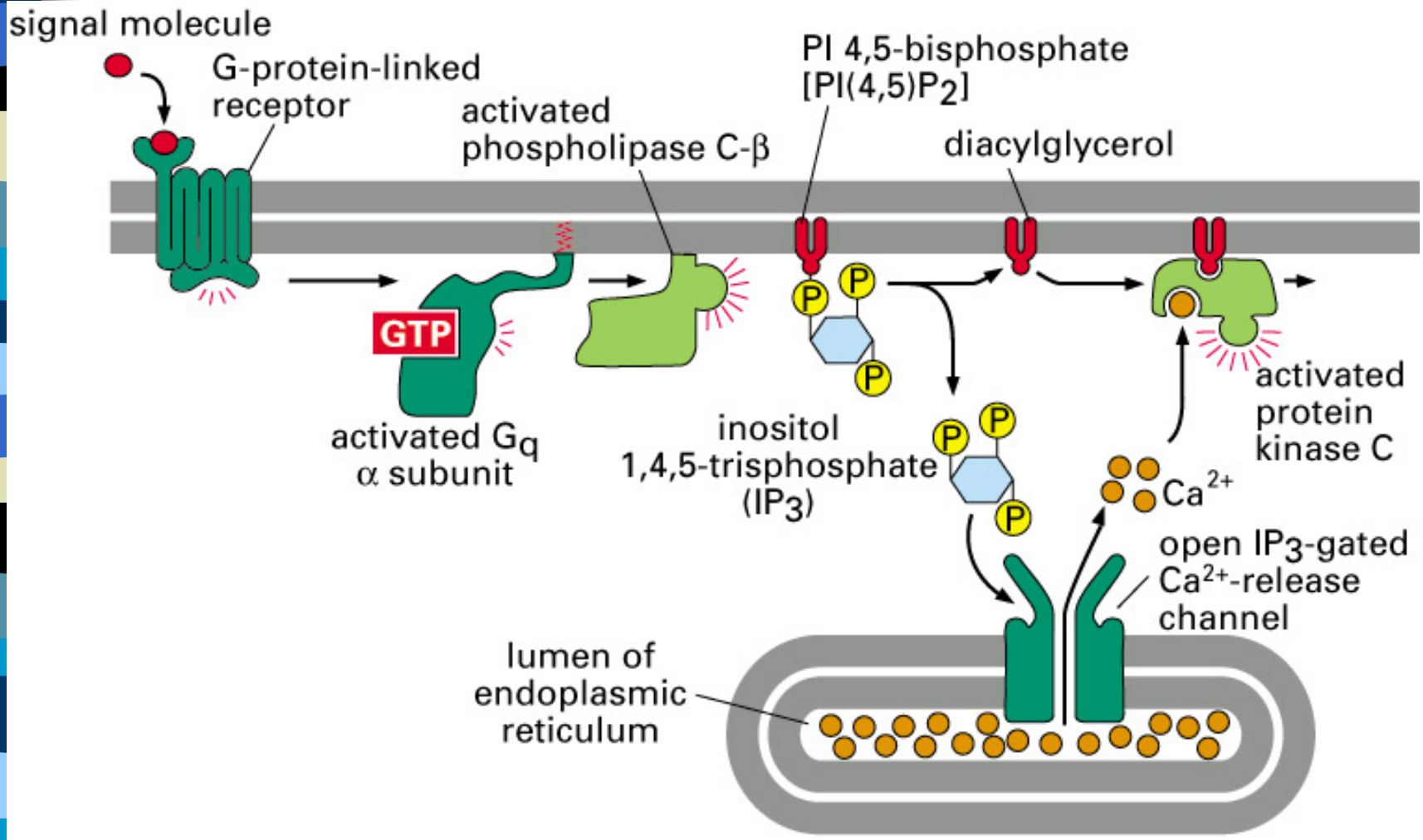


Figure 15–36. Molecular Biology of the Cell, 4th Edition.

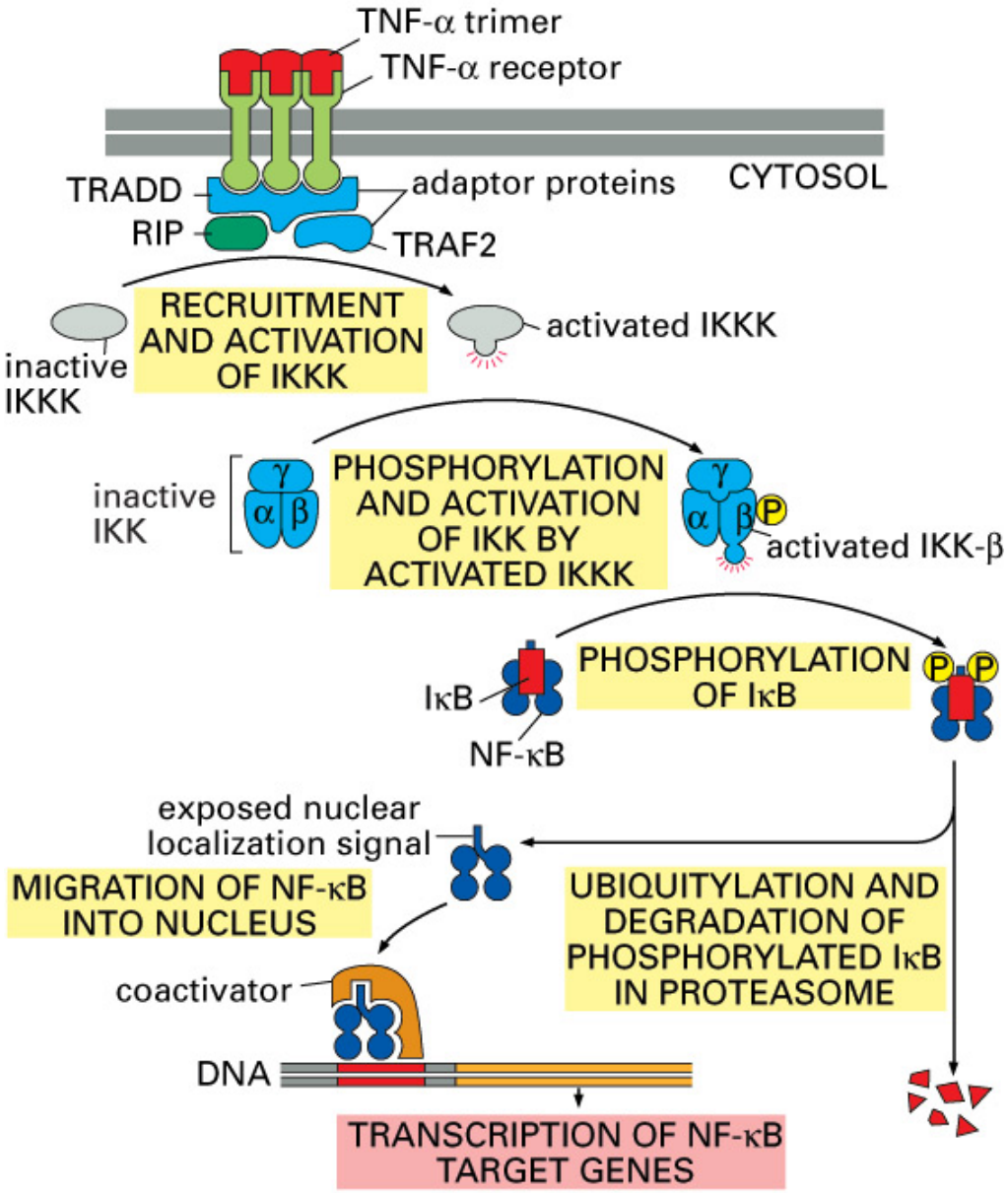


Figure 15–74. Molecular Biology of the Cell, 4th Edition.

Protein (1):

- identifikace (specifická protilátka; MALDI-TOF; microsequencing)
- zařazení (práce s databázemi)
- regulace exprese (analýza exprese v modelovém buněčném systému a in vivo)
- lokalizace v buňce (live cell imaging; imunofluorescenční techniky; subcellular fractionation)
- interagující proteiny (imunoprecipitace; GST-pull-down; yeast two-hybrid screen)
- in vitro funkce – enzymatická aktivita?, strukturní protein?, membránový protein?, adaptérový protein?, receptor?;
- in vivo funkce (transgenní modely, tkáňově specifická exprese, mutace)

Protein (2):

- funkce jednotlivých domén;
- posttranslační modifikace (fosforylace, acetylace, glykosylace)
- regulace odborávání proteinu (ubikvitinace; proteazomální degradace)
- DNA interakce, transkripční aktivace, identifikace cílových genů, DNA-binding sites (EMSA, reporter gene assays)
- manipulace s proteinem (overexprese; dominant negative constructs, antisense oligonucleotides, siRNA)
- proteomika
- protein v buněčném kontextu
- izolace a purifikace

SDS-PAGE a Western blotting, 2-D elektroforéza

SDS-PAGE Gel Electrophoresis

- Separation based (predominantly) on mass of protein.
- SDS denatures and coats proteins. Because SDS is negatively charged all proteins have a constant charge to mass ratio and migrate based on mass.
- Some proteins migrate abnormally.
- Native gel electrophoresis is similar but without SDS. In native electrophoresis protein migrate based on their charge and mass and can move toward either electrode.

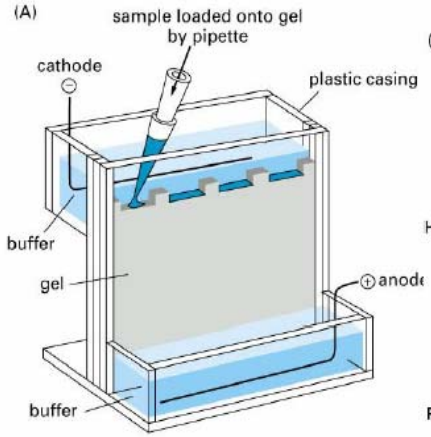


Figure 8–14 part 1 of 2. Molecular Biology of the Cell, 4th E

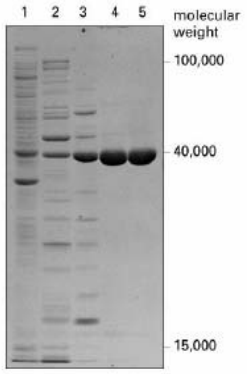


Figure 8–15. Molecular Biology of the Cell, 4th Edition.

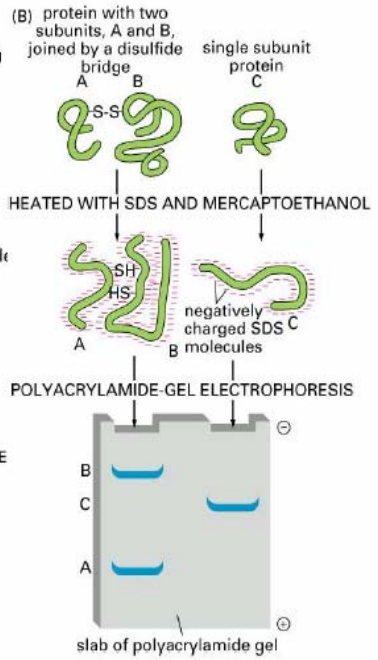
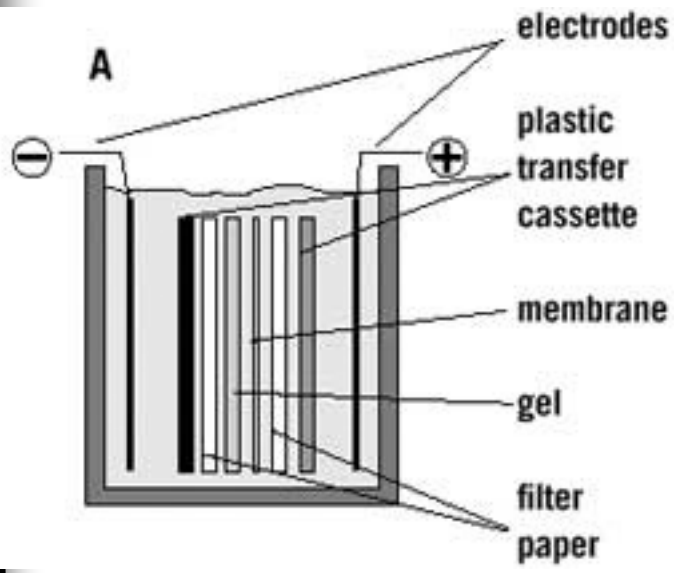
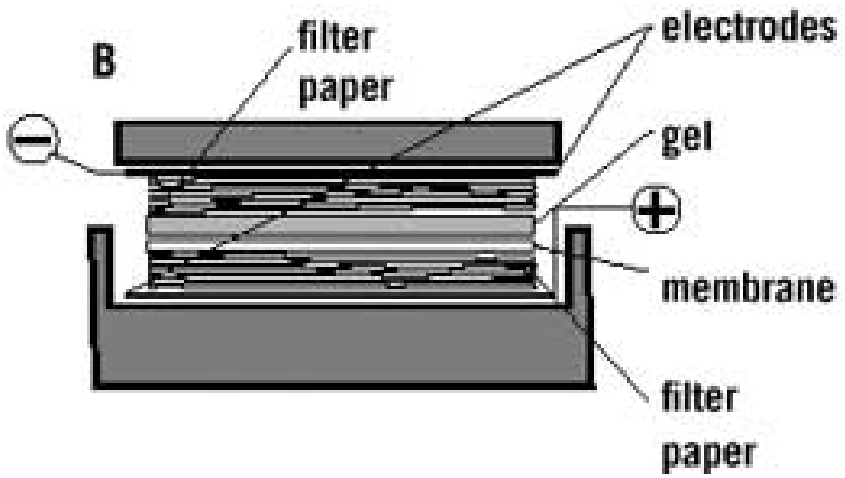


Figure 8–14 part 2 of 2. Molecular Biology of the Cell, 4th Edition.

Western blotting – transfer:

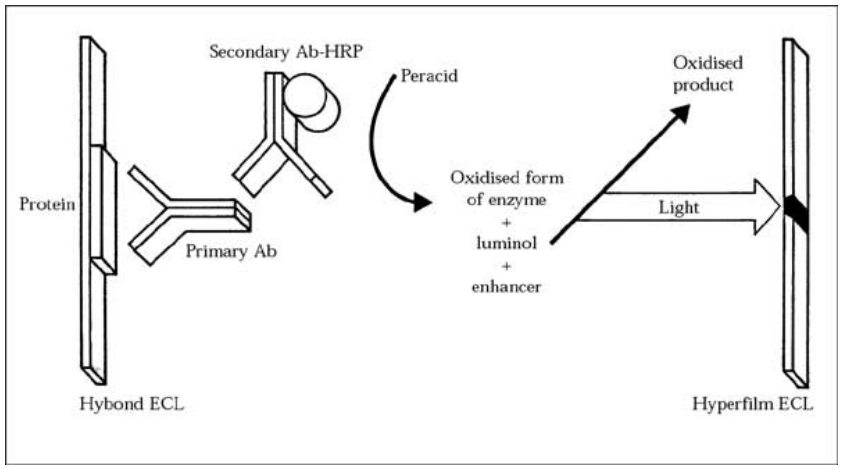




Membrány:

- **PVDF**
- **nitrocelulóza**

Western blotting – imunodetekce:



Western blotting is used to detect a target protein in a sample (containing a complex mixture of proteins) by using a polyclonal or monoclonal antibody specific to that protein.

Western Blot Procedure

• Blocking

The membrane is blocked, in order to reduce non-specific protein interactions between the membrane and the antibody. This is achieved by placing the membrane in a solution of bovine serum albumin (BSA) or non-fat dry milk (NFDM).

• Primary Antibody

The first antibody to be applied (specific for protein of interest) is incubated with the membrane. The antibody is diluted in a buffer solution (PBS) containing a carrier protein (BSA or NFDM) along with some detergent. The primary antibody is specific for the protein of interest, and, at appropriate concentrations, should not bind any of the other proteins on the membrane.

• Secondary Antibody

After rinsing the membrane to remove unbound primary antibody a secondary antibody (figure 3 – green antibody) is incubated with the membrane. It binds to the primary antibody. This secondary antibody can be linked to an enzyme that allows for visual identification by producing vissible changes in colour, chemiluminescence or fluorescence. An alternative is to use a radioactive label.

• Developing

The unbound secondary antibodies are washed away, and the enzyme substrate is incubated with the membrane so that the positions of membrane-bound secondary antibodies will emit light. Bands corresponding to the detected protein of interest will appear as dark regions on the developed film. Band densities in different lanes can be compared providing information on relative abundance of the protein of interest.

1.The first paper describing the use of electrophoresis to transfer proteins from a gel to a membrane. Towbin H., Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications.Proc Natl Acad Sci U S A. 1979 Sep;76(9):4350-4

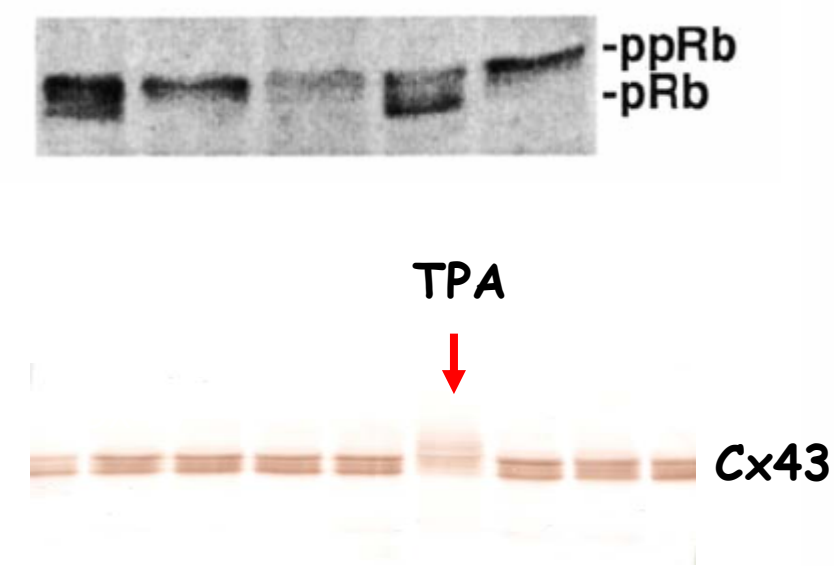
2.The classic paper by UK Laemmli - the most cited article in the journal's history. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970 Aug 15;227(5259):680-5.

3. The first paper to use the term western blot. Burnette WN. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem. 1981 Apr;112(2):195-203

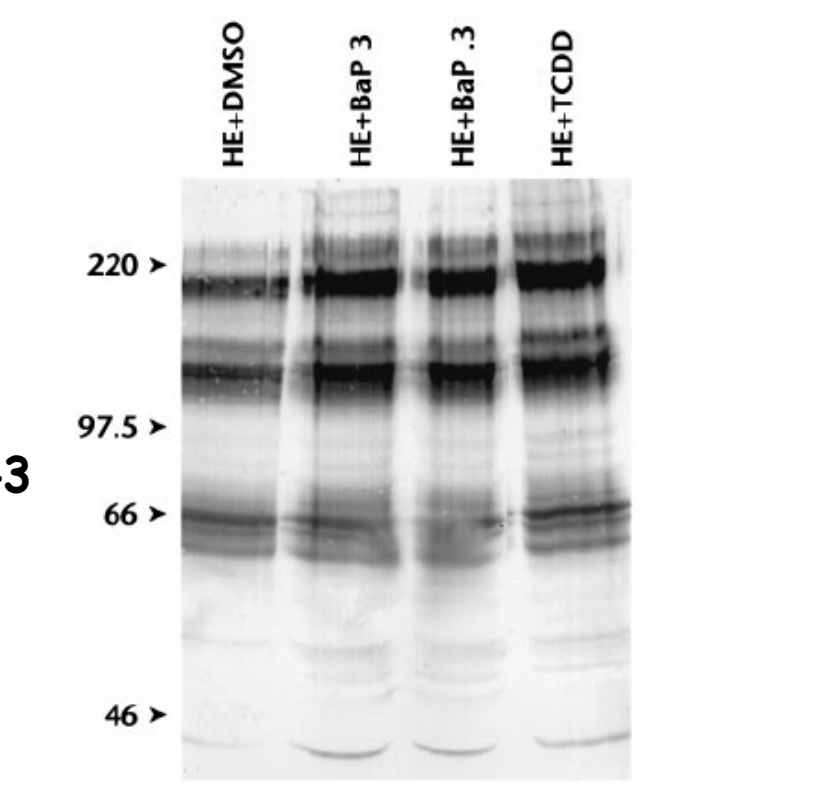
http://www.westernblotting.org/

Modifikace

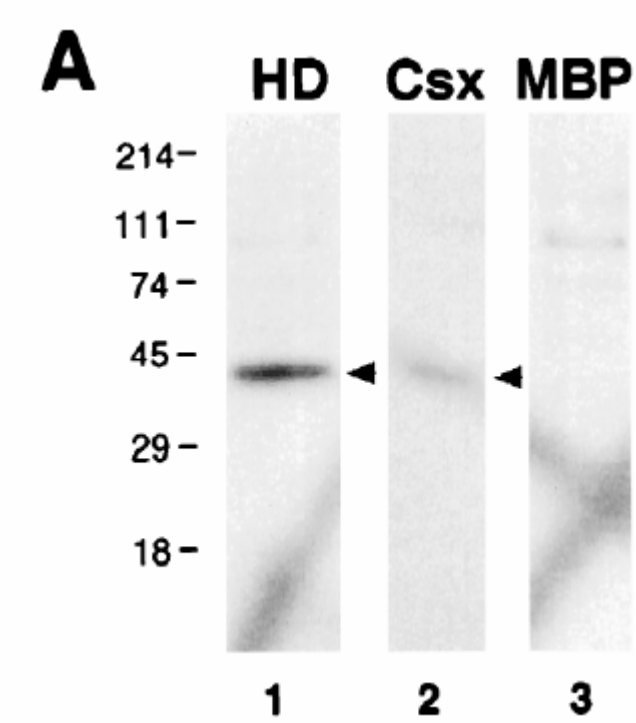
•gel-shift assays



• whole cell protein modification assays (e.g. tyrosine phosphorylation)



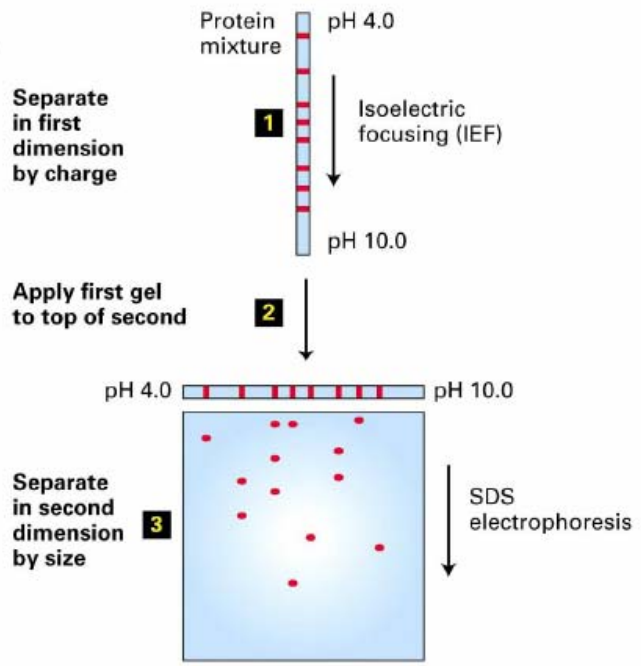
Elektroforetická separace může být využita i pro funkční analýzu proteinů:

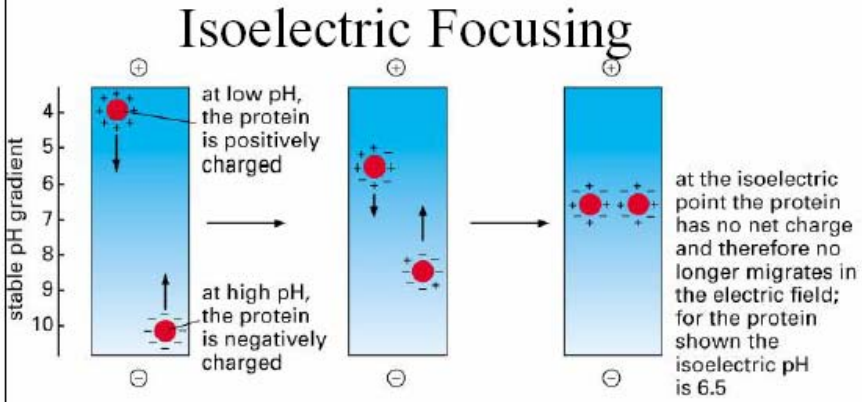


In-gel kinase assay. MBP (0.2 mg/ml), and MBP-Csx/Nkx2.5 or MBP-HD fusion protein (0.1 mg/ml) were incorporated into SDS–15% PAGE separation gels. NIH 3T3 nuclear extracts (50 mg) were boiled in SDS sample buffer and then loaded on the gels. After SDS-PAGE, gels were washed twice in 20% isopropyl alcohol for 30 min, denatured with 6Mguanidine-HCl in the buffer (40 mM HEPES [pH 7.4], 20 mM NaCl) for 60 min, and renatured gradually by decreasing Renatured gels were incubated in the presence of [g-32P]ATP (0.25 mCi/ml)–25 mM ATP in 40 mM of HEPES (pH 8.0)–5 mM MgCl2–2mMDTT–0.1mMEGTA at room temperature for 1 h and washed with 5% trichloroacetic acid and 1% sodium pyrophosphate.

2D-Gel Electrophoresis

- 2D-gel electrophoresis allows separation of "all" of the proteins in a cell.
- Separates by isoelectrofocusing in the first dimension (separates based on isoelectric point) followed by separation based on mass (SDS-PAGE).
- Can be used to examine changes in protein levels between different cell types or different cell states.
- Mass spectrometry can be used to identify protein in a spot.





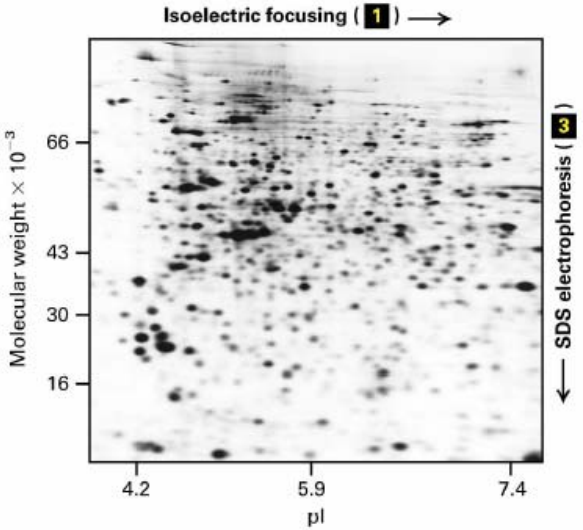


Figure 8–16. Molecular Biology of the Cell, 4th Edition.

Protein Fingerprinting

- Proteins can be identified by protein fingerprinting.
- A protein is fragmented using proteolytic enzymes or chemical cleavage.
- Fragments are then separated by electrophoresis and chromatography to give a fingerprint (classical procedure).
- Currently fragments are analyzed by mass spectrometry and proteins identified by comparison to protein data base.

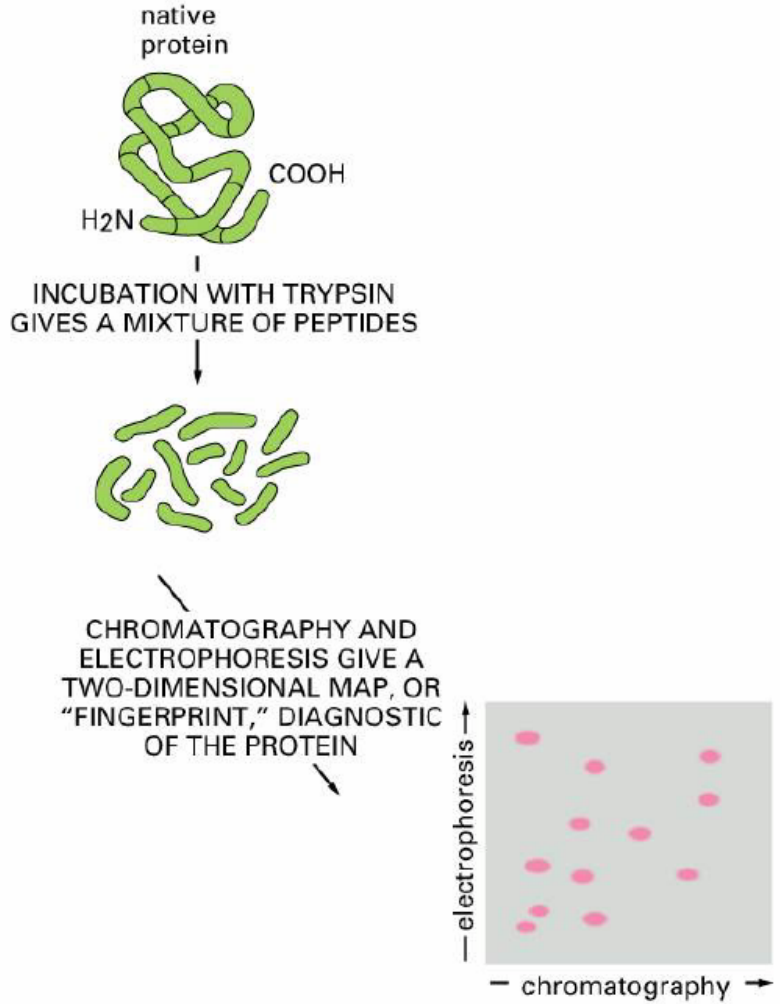
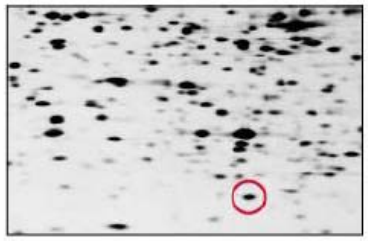


Figure 8–19. Molecular Biology of the Cell, 4th Edition.

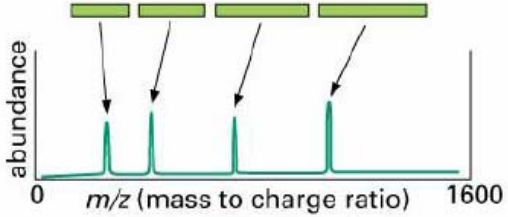
Identifying proteins by Mass-spectrometry



single protein spot excised from gel

N C

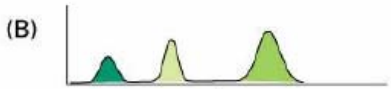
PEPTIDES RELEASED BY TRYPTIC DIGESTION AND THEIR MASSES MEASURED USING MALDI-TOF MASS SPECTROMETRY



PROTEIN SEQUENCE DATABASES SEARCHED FOR MATCHES WITH THEORETICAL MASSES CALCULATED FOR ALL TRYPSIN-RELEASED PEPTIDES

IDENTIFICATION AND ISOLATION OF CORRESPONDING GENE

Figure 8–20 part 1 of 2. Molecular Biology of the Cell, 4th Edition.



single protein peak from column chromatography

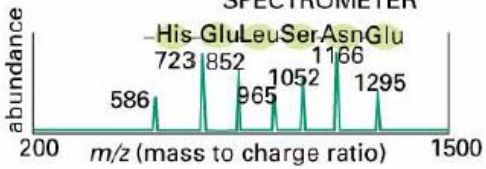
N C

PEPTIDES PRODUCED BY TRYPSIN DIGESTION OF PURIFIED PROTEIN

FIRST MASS SPECTROMETER GIVES PEPTIDE MASSES, AS IN (A)

EACH PEPTIDE IS FURTHER FRAGMENTED AT PEPTIDE BONDS

MASSES OF FRAGMENTS MEASURED ON A COUPLED SECOND MASS SPECTROMETER



THE MASS DIFFERENCES BETWEEN FRAGMENTS CAN BE USEDTO CONSTRUCT A PARTIAL AMINO ACID SEQUENCE. THE DATAMAY ALLOW A GENE IDENTIFICATION, OR PROVIDE THE MEANS FOR CLONING THE GENE

Figure 8–20 part 2 of 2. Molecular Biology of the Cell, 4th Edition.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 278, No. 50, Issue of December 12, pp. 50781–50790, 2003
Printed in U.S.A.

Use of Proteomics to Demonstrate a Hierarchical Oxidative Stress Response to Diesel Exhaust Particle Chemicals in a Macrophage Cell Line*

Received for publication, June 17, 2003, and in revised form, September 27, 2003
Published, JBC Papers in Press, September 30, 2003, DOI 10.1074/jbc.M306423200

Gary Guishan Xiao‡§, Meiying Wang§, Ning Li§, Joseph A. Loo‡, and Andre E. Nel§¶

From the ‡Keck Functional Proteomics Center, Department of Biochemistry and Molecular Biology and the §Department of Medicine, Division of Clinical Immunology and Allergy, David Geffen School of Medicine, UCLA, Los Angeles, California 90095

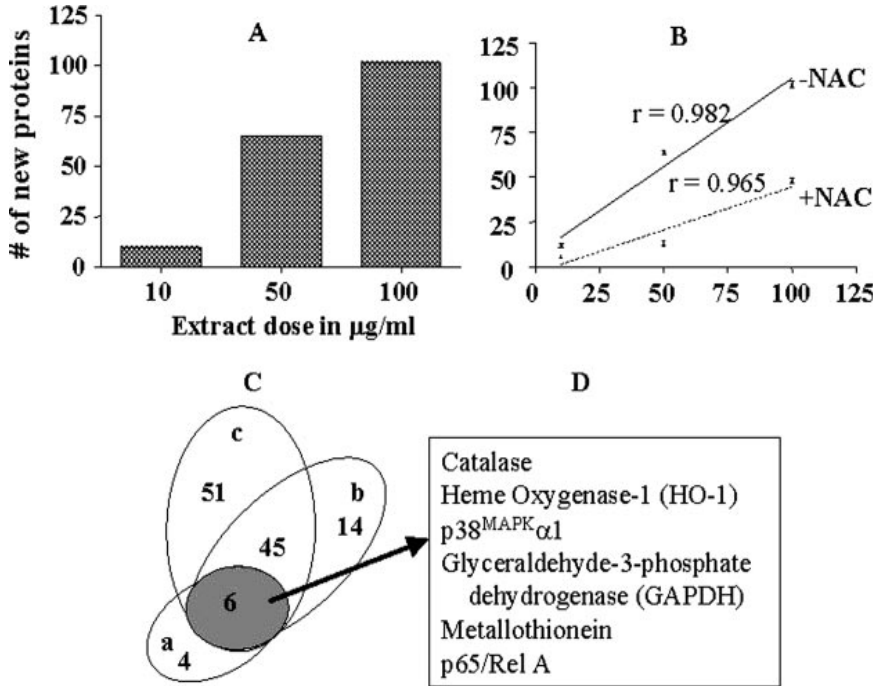


FIG. 4. **Dose-dependent increase in new protein expression in RAW 264.7 cells as determined by two-dimensional gels.** *A*, dose-dependent increase in new protein expression in response to organic DEP extracts; protein expression was suppressed by NAC. *B*, regression analysis showing the linear correlation between extract dose and the number of newly expressed proteins. *C*, Venn diagram to show the overlapping and unique expression profiles at different doses of the DEP extract, *D*, list of six new proteins induced at all extract concentrations. RAW 264.7 cells were exposed to DEP extracts at indicated concentrations, in the absence or the presence of 20 mM NAC, for 6 h before cellular extraction and analysis of the soluble proteins by two-dimensional electrophoresis. These data were reproduced three times, during which the variability in protein expression was 10%.

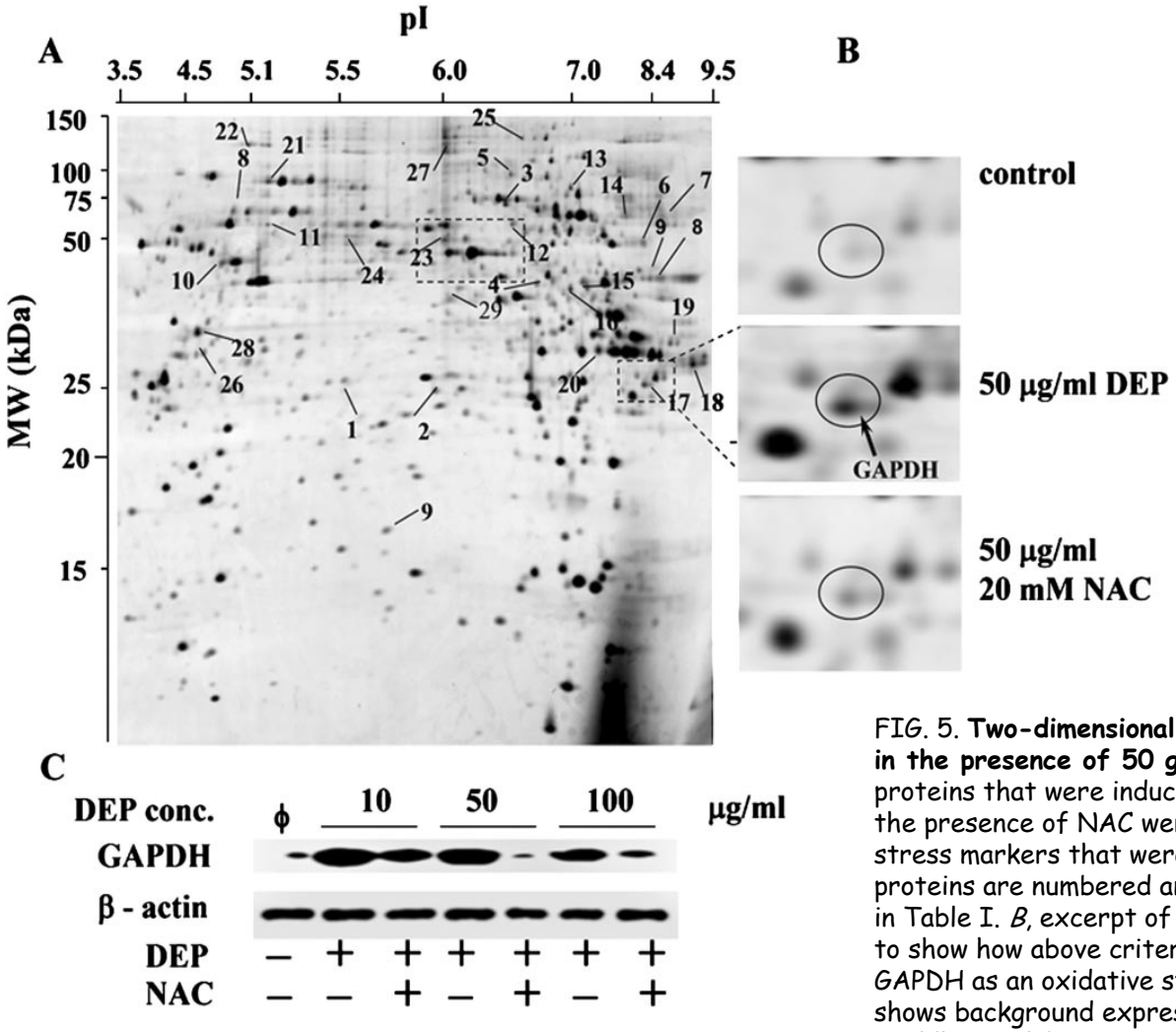


FIG. 5. **Two-dimensional gel electrophoresis profile in the presence of 50 g/ml organic DEP extract.** *A*, proteins that were induced 8-fold and subtracted in the presence of NAC were selected as oxidative stress markers that were identified by MS. Those proteins are numbered and their identities disclosed in Table I. *B*, excerpt of the two-dimensional profile to show how above criteria led to the identification of GAPDH as an oxidative stress marker. The *top panel* shows background expression in untreated cells, the *middle panel* shows increased expression by the extract, and the *bottom panel* shows the subtracted response in the presence of NAC. *C*, GAPDH immunoblotting shows the subtractive expression of this protein in crude cell lysates. See "Materials and Methods" for experimental details. These data were reproduced three times, during which the variability in protein expression was 10%.

TABLE I

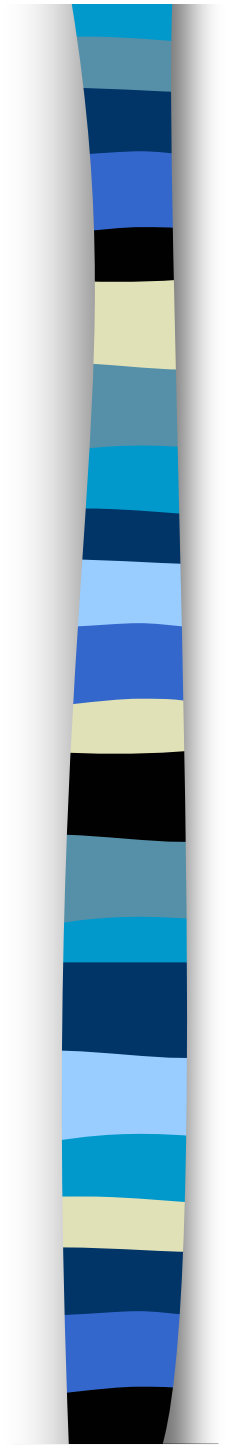
Protein assignments from whole RAW 264.7 cells exposed to both 50 and 100 μg/ml DEP extracts

*, Pro-inflammatory, the potential to contribute to biological events culminating in or regularly inflammation; oxi, oxidative.

Protein assigned	Spot no.	DEP dose[a]	Protein Data Bank code	Observed M_r	Observed pI value	NAC suppressibility[b]	Sequence coverage (MS)	Possible oxidative stress role
		μg/ml		kDa			%	
Heme oxygenase-1	23	≥10	P14901	42.0	6.05	++++	38	Cytoprotective/ARE-driven
Catalase	12	≥10	P00432	57.5	6.40	+++	66	Cytoprotective/ARE-driven
Metallothionein	18	≥10	BAB24517	23.2	9.20	+++	73	Cytoprotective/oxi stress-inducible (46–48)
Glyceraldehyde-3-phosphate dehydrogenase	17	≥10	P16858	35.0	8.40	+++	26	Increased abundance but decreased function with oxi stress (56, 57, 66)
Nuclear factor κB (Rel A)	2	≥10	P98150	23.4	6.05	++++	18	Anti-apoptic/pro-inflammatory (54)
$p38^{MAPK}$ α1	24	≥10	Q99MG4	41.4	5.60	+++	13	Pro-inflammatory
Granulocyte/macrophage colony-stimulating factor precursor	9	≥50	P01587	16.1	5.80	++	13	Pro-inflammatory (55)
Tumor necrosis factor receptor 2	29	50 only	P25119	50.3	6.62	++++	13	Pro-inflammatory (56)
Putative Rho/Rac guanine nucleotide	27	≥50	P52734	106.0	6.2	++	14	Pro-inflammatory (71)
Early growth response protein 4 (EGR-4)	4	≥50	Q00911	49.6	6.70	++	12	Transcriptional cytokine inducer (57)

http://www.pdb.org/pdb/home/home.do

http://www.ncbi.nlm.nih.gov/sites/entrez



Protein (1):

·regulace exprese (analýza exprese v modelovém buněčném systému a in vivo)
· lokalizace v buňce (live cell imaging; imunofluorescenční techniky; subcellular fractionation)
· interagující proteiny (imunoprecipitace; GST-pull-down; yeast two-hybrid screen)
· in vitro funkce – enzymatická aktivita?, strukturní protein?, membránový protein?, adaptérový protein?, receptor?;