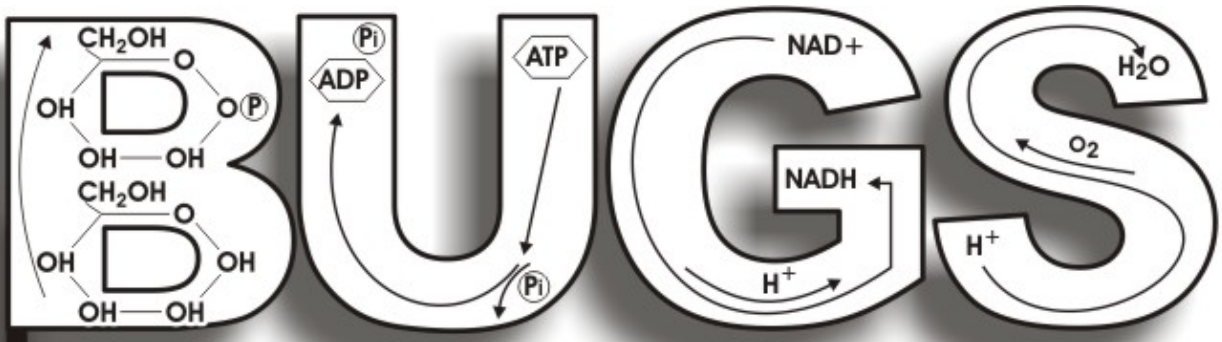

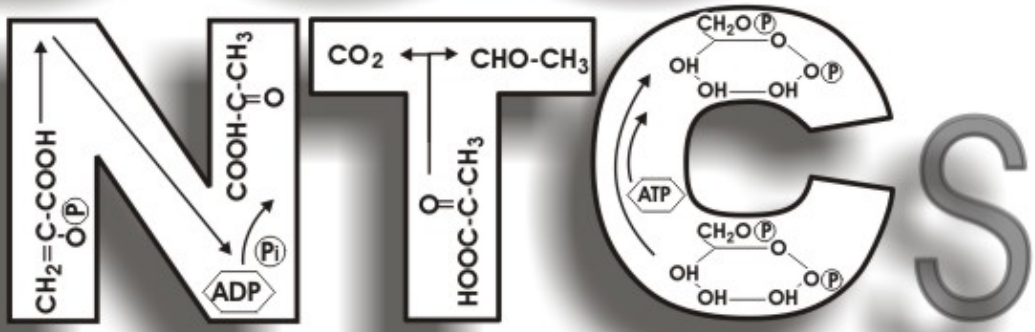

BIOC 312

SET # 9 Lectures 30- 31 (03/28/07 –03/30/07)

ANNOUNCEMENTS

ALL NEW NTC SETS FOR THIS CLASS WILL NOW BE POSTED ONLINE, MAKE SURE YOU HAVE ACTIVATED YOUR ATHENA ACCOUNT!!!

GOOD LUCK STUDYING!

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03/28/2007

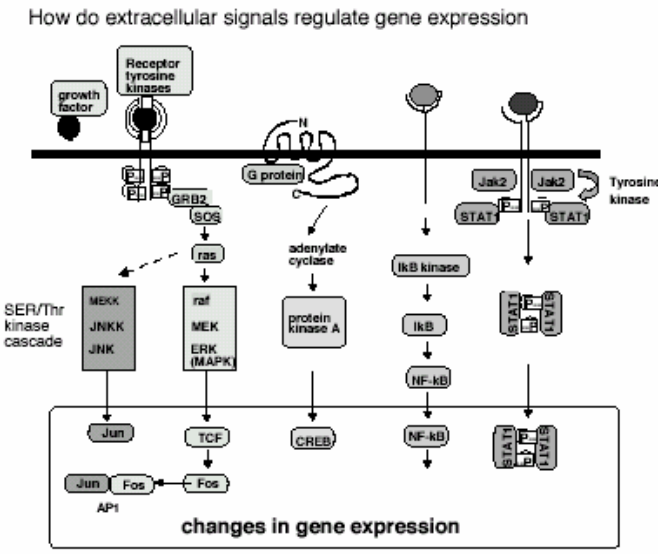
Lecture #30–Protein Phosphorylation

NOTE: This NTC is meant to be used as a study aid to supplement your own class notes. Hence, not all of the text or diagrams contained in the lecture slides will be reproduced here.
Please send any comments or questions about NTCs to either Misha or James through e-mail at **bugs_u2reps@yahoo.ca**

- Review from Biochemistry 212: We had discussed how extracellular signals can initiate a signal from the outside of the cell into the cell. This results in changes in gene expression in the nucleus.
- In the following two lectures, we will work out how we get from the cell surface into the nucleus. We will learn a bit about the structure of tyrosine kinases and their activation. We will also talk about other types of signaling events that result in changes in gene expression.
- The focus of these two lectures will be on the change in gene expression in the nucleus and a bit of the structure. You **DON'T** need to swallow up in detail all of the signaling pathways.

Tyrosine kinases:

- phosphorylates SPECIFICALLY tyrosine residues and are **transmembrane** proteins
- as an enzyme, they are **inactive** in the membrane
- When the ligand binds to the receptor, it promotes the dimerization of the receptor. This event changes the conformation of the kinase so that it can now bind ATP
- Once it binds ATP, it hydrolyzes the trans phosphate from the gamma residue on the ATP to tyrosine residues within the receptor.
- Proteins that contain SH2 domains get recruited to tyrosine phosphorylated residues outside the kinase domain – this is in a **SEQUENCE SPECIFIC manner**; they recognize the phosphorylated tyrosine in the context of the surrounding amino acids (don't need to remember that for these lectures!)
- One of the proteins that was recruited to the receptor was an adaptor protein - it recruits an exchange factor that promotes the activation of ras
- ras is usually in a **GDP bound** form in the membrane; it has a high affinity for GDP
- It cannot release GDP, but when it interacts with an exchange factor, **SOS,** it changes the conformation of RAS so that it releases GDP



- GTP is present in concentrations 10x higher in the cell, so GTP will bind to the pocket in ras – so this is really an exchange of GDP for GTP (**Remember:** this is NOT phosporylation of the GDP!!!)
- Once ras is bound to GTP, it changes conformation; it activates a signaling module (serine/threonine kinases – there are three)
- These bind to a scaffold protein; you always activate the raf serine/threonine kinase, which in turn will activate the MEK serine/threonine kinase, which in turn will activate the ERK (or MAPK = MAP kinase)
- Once it is phosphorylated and activated, it will enter the nucleus (this end point is our focus).
- There are other types of serine/threonine kinase cascades. There is another one that will result in the activation of another MAP-like kinase called JNK (also goes into the nucleus).
- In the nucleus, each of these serine/threonine kinases will phosphorylate a different transcription factor.

G-protein Coupled Receptors

- They can activate two major pathways:
  - adenylate cyclase, that would release cAMP, which will activate different kinases, one of those being **protein kinase A** (a serine/threonine kinase) – it enters the nucleus, and phosphorylates a protein called CRE Binding protein (**CREB** – another transcription factor). These types of receptors are transmembrane proteins. They themselves do NOT have intrinsic kinase activity, but they associate with serine/threonine kinases that are inside the cell.
- **Bottom line**: When you activate these, you will activate a kinase!
- One of these is another serine/threonine kinase called **IkB kinase**. We will talk about the regulation that results in the release of a transcription factor called **NF-κB** that actually sits in the cytoplasm. Once it is released, it can enter the nucleus.

- There are multiple ways through which we are regulating transcription, all of which are being regulated at the cell surface.

Cytokine Receptors

- Have no enzymatic activity
- They associate with a tyrosine kinase called Jak2 (don't need to remember the name).
- These receptors can interact with a transcription factor
- A transcription factor called **STAT1.**
  - It becomes tyrosine phosphorylated. Once this occurs, it enters the nucleus.
- The signal here has no intermediate at all. It goes straight from the receptor, the transcription factor is released and shoots straight into the nucleus.
- These types of changes in transcription can be activated within minutes after stimulating that cell.
  - We see activation of signaling pathways within 2-5 minutes and we will start to see changes in transcription about 30 minutes post-stimulation.
  - This can be seen in the presence of **cycloheximide**, a drug which will prevent new protein synthesis. This fact tells us that all the proteins that are required for the initial changes in gene expression are actually present in the cell at the time that you stimulate that growth factor receptor.
  - You will require new protein synthesis to generate some of the products that are induced by these genes - they are required to induce other genes that are required for the full biological program of that growth factor.

Activation of Receptor Tyrosine Kinase by Ligand

- Tyrosine kinases have a **kinase domain** – these domains are highly conserved both between tyrosine kinases and serine/threonine kinases.
- Tyrosine kinases contain a tyrosine in the kinase domain
- In the absence of growth factor, it is inside the membrane, inactive as an enzyme. When you add the growth factor, you promote dimerization of the receptor.
- The first event is the phosphorylation of the tyrosine within the kinase domain.
- The dimerization induced by ligand binding promotes a conformational change so that ATP can bind; the first tyrosine to get phosphorylated is in the kinase domain. This kinase is now active, releases ADP where ATP will bind.
- The next events are that you will phosphorylate tyrosines outside the kinase domain.

*** Remember: Not all tyrosines will get phosphorylated, but it is ALWAYS the same tyrosines that will be phosphorylated in each receptor tyrosine kinase. ***

- Those tyrosines that are phosphorylated provide specific binding sites for SH2 domain containing proteins. Some of these will become tyrosine phosphorylated by the receptor. Others will not, and will just be recruited to the membrane.
    - There are approximately 250-270 amino acids.

Diagrams (pg 2 of handouts)

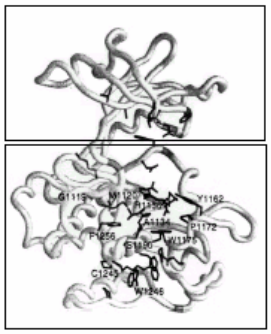
First: aligns the sequence of the insulin receptor kinase – a receptor tyrosine kinase for insulin (this is the receptor that you will get mutations in that will cause diabetes); regulates metabolism in response to insulin.
Second: Epidermal Growth Factor (EGF) receptor.
Third: Platelet Derived Growth Factor
Fourth: Fibroblast Growth Factor Receptor – these are produced at sited of wounds. They recruit fibroblasts and stimulate proliferation and migration.

- Essentially all the kinase domains at the sequence level show structural similarity; they have specific motifs
- Two other tyrosine kinases, SARK and ABL → only associated with the membrane (NOT transmembrane proteins)
- Serine/threonine kinase: Protein Kinase A (a.k.a. cyclic AMP dependent kinase); this is the kinase that is activated downstream from the G-protein coupled receptors that activate adenylate cyclase
    - There are regions that are highly conserved in all tyrosine kinases
    - All of the highly conserved domains form structural domains that are required for function of the enzyme
    - All of these proteins have similar structural domains. The stuff in between them can be quite variable but when you look at the crystal structure of all of these enzymes you can essentially superimpose all of the crystals one on top of the other
- 1st Conserved region - **Glycine rich loop –** this is the ATP binding pocket
    - Downstream from this, always at the same distance, there is an invariant lysine residue – critical for the phospho transfer of the gamma phosphate from ATP to the substrate. This can be the kinase receptor itself or one of the signaling proteins that is recruited to the receptor (through the SH2 domain, for example).
- There is a highly conserved pocket of amino acids that forms a **catalytic loop** – this can differ from different tyrosine kinases; you can have an AAR or an RAA.
- Serine/threonine kinases have a specific motif (KPE) – it contains a catalytic base, which is required for the function of the enzyme
- Activation loop → this is the domain where in tyrosine kinases, you always have a tyrosine present
    - Some tyrosine kinases have two tyrosines here (for example. The insulin receptor tyrosine kinase) as opposed to, for example, the EGF receptor
    - Serine/threonine kinases always have a serine or a threonine residue at this position
    - Essentially, phosphorylation of the tyrosine or the serine and threonine residue have the same effect
    - What you are doing is adding a charged phosphate residue onto that tyrosine or serine/threonine – this will have an impact on the folding and the structure of the enzyme

Crystal structure of Protein Kinase A

- Can essentially superimpose on the crystal structure of the Insulin receptor (very, very similar!)
- When you look at the crystal structure, the kinase domain folds into two main domains:
    1. **the amino terminal domain –** contains the ATP binding-site, the lysine rich domain and the invariant lysine residue that is required for the phospho transfer. The phosphate from the ATP that binds is going to project down towards the catalytic lobe (the C terminal lobe)

The 3 dimensional structure based on PKA (ser/thr) and Insulin receptor (Tyr) K



- Kinase domain is composed of two lobes
- N terminal lobe
  - ATP binding site Gly rich - invarient Lys is involved in the phosphotransfer of γ phosphate from ATP.
- C terminal lobe
  - contains the catalytic loop.

2. **the C terminal domain** – the catalytic pocket is buried in here. It is in this pocket that you promote the transfer of phosphate from the gamma phosphate of ATP. The tyrosine residue that is present in the kinase domain is on a loop.

In some cases, they will crystallize the receptor either in the presence of an inhibitor (ex. an ATP analog that can't be hydrolyzed) or in the presence of a phosphorylated peptide

Bottom Line: In each case, what you are seeing is the projection of the phosphate into the catalytic pocket.

- If you look at the inactive conformation, the activation loop is essentially folded over and the tyrosine reside that is non-phosphorylated is projecting into the catalytic pocket
- In the inactive conformation, the catalytic pocket is filled by the tyrosine residue. You cannot bind ATP into the ATP binding site.
- When you activate the enzyme by binding ligand to the receptor or by activating protein kinase A, what you are doing is inducing a conformational change. You get a rotation of the amino terminal lobe with the carboxy terminal lobe. This opens up the kinase such that you can now bind ATP. This opens up the catalytic domain such that a peptide with a tyrosine residue can enter into the catalytic domain.
  - The 1st event on binding ATP is that you phosphorylate the receptor itself on the tyrosine residue(s) in the activation loop.
  - The charge from the phosphate forms electrostatic repulsion such that it projects the loop out; this causes some of the AA in the loop to form specific interactions with other AA on the body of the C terminal lobe.
- Remember: Some of the tyrosines in the C-terminus of the receptor will get phosphorylated.

Dimer of the receptor: the C-terminus will fold up into the catalytic pocket of the adjacent kinase. You are essentially trans-phosphorylating these receptors. This is another reason why you have to dimerize them for activation.

- Tyrosines that are phosphorylated outside the kinase domain on the receptor itself can bind proteins that contain an SH2 domain; some of those can be phosphorylated by the receptor.
- Once the tyrosines are dephosphorylated, that activation loop begins to collapse back into the catalytic domain, essentially occluding the binding of substrates or the receptor itself to that domain.
- Receptors are usually catalytically active for about 15-30 minutes after you stimulate them. There are many mechanisms through which you down-modulate them, only one of them being through dephosphorylation.

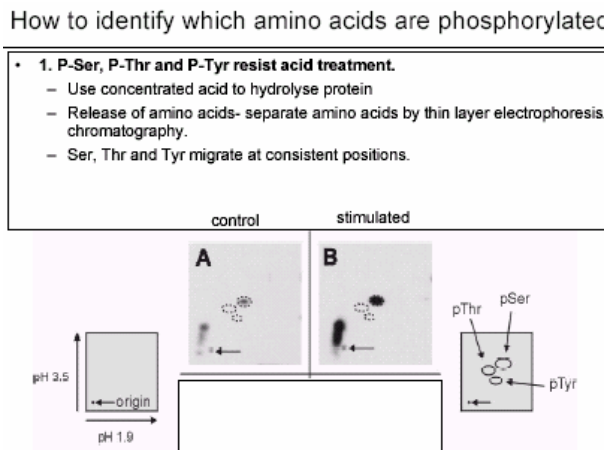
***Question*: How do you identify whether your protein is a serine kinase or a tyrosine kinase?**

- One way is to establish which AAs can be phosphorylated by that protein in vitro or in vivo.
- In vitro, you would add radiolabeled gamma ATP (gamma phospate is radioactive)
- In vivo, you add radioactive phosphate; this is just free phosphate. You can add it to cells in culture, or tissues (animals).

If you radioactively label your cells with inorganic phosphate, you can stimulate with growth factor. You can also purify your receptor tyrosine kinase (but at that time you don't know it's a tyrosine kinase) or you can purify your protein of interest.

You can also have your kinase which you can synthesize in bacteria (in vitro), you can purify that and then you can just add radioactive gamma labelled ATP and do an **in vitro kinase assay.** These enzymes will function in vitro in the presence of ATP at the right salt concentration.

- Phosphoserine, phoshpothreonine and phosphotyrosine are RESISTANT TO ACID TREATMENT.
- If you treat a protein with concentrated acid, it will essentially hydrolyze the peptide bond, but it does not hydrolyze the phosphate bond.
- If you release free AA, you will release the phosphorylated tyrosine, phosphorylated serine and phosphorylated threonine residues in that protein.



- You can perform **thin layer chromatography** – you essentially dry down your hydrolyzed protein, and spot it at the line of origin. You separate the amino acids into two dimensions, based mostly on the pH.

Diagram: Run one side at pH 1.9, then turn around and let run at pH 3.5 (2D separation).

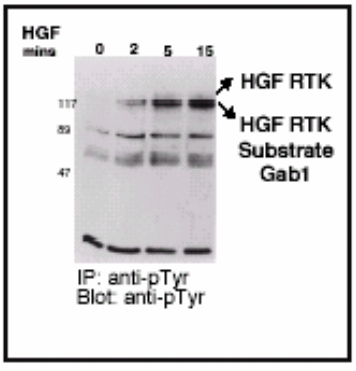
- This separates the AAs, and you will see the radioactive spots when you develop the gel.
- Important to run standards on the TLC plate. They always migrate with a consistent pattern.
- Looking at the control group in the diagram, we see some phospho-serine in the protein that you purified.
- If you stimulate cells with growth factor, you see a large increase in phosphorylation, but only on the serine residue (in this case).

Partial Alkali Hydrolysis → in this case the protein is still intact, but this tends to remove phosphate from serine and threonine residues but will permit the retention of phosphate on tyrosine residues (stronger bond).

- Can treat the whole gel with partial alkali hydrolysis
- Can look before treatment, seeing which proteins are phosphorylated
- Can then look after hydrolysis and see which proteins lose phosphate
- If you retain the phosphate, then you are looking at a tyrosine phosphorylation site on that protein.
    - This doesn't tell you that it is a kinase! However, it tells you that the protein is phosphorylated on tyrosine

Immunoblotting → can make antibodies that will specifically recognize a **phosphorylated** tyrosine residue.

- The 1st type of antibodies that were made were created using two synthetic phospho peptides, where the tyrosines residues are phosphorylated.
- You inject with this material rabbits, & they generate an immune response and the antibodies may be purified, some of which will recognize phosphorylated tyrosine residues.
- Those antibodies can be used to precipitate out proteins of interest (in the example on the left, we have activated our receptor tyrosine kinase with its ligand, **HGF**) and we run out all of the proteins.



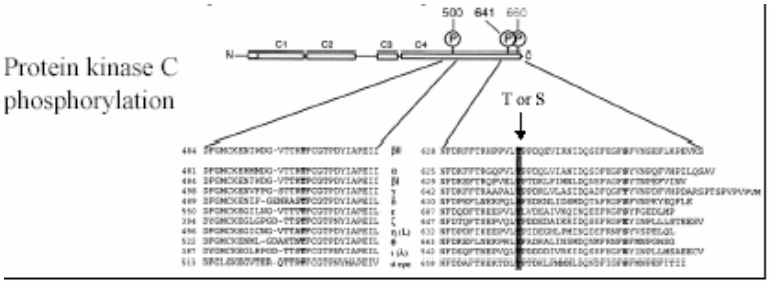
- First, you immunoprecipitate with antibody to phosphotyrosine. You transfer that to nitrocellulose or different membrane, and you blot with anti- phosphotyrosine.
- In the absence of stimulation, there is very little phosphorylated phospho-tyrosine proteins in these cells that are detected with this antibody.
- Stimulate for 2 minutes - you begin to see an increase in phosphorylated proteins
- By fifteen minutes, we see high levels of phosphorylated protein in the cell
- We can see two specific proteins:

1. The top one, which is very faint, corresponds to the receptor tyrosine kinase (HGF RTK)
2. Seen as a big blob; corresponds to a substrate of the receptor. The reason why this band is so intense is because this protein gets phosphorylated on about ten tyrosine residues by the receptor tyrosine kinase.

- Using this strategy, when we stimulate cells with our growth factor and activate our receptor, we see predominantly phosphorylation of this protein that we know is a protein called **Gab1**.
- We can stimulate the same cells with another growth factor, and we may see a completely different pattern of tyrosine phosphorylated proteins; that would tell you that that receptor activates different downstream signaling proteins.
- We used this strategy at the time to actually purify this protein using anti-phosphotyrosine columns (make column with the anti-phosphotyrosine antibody) – you pass the whole cell lysate onto that column. Proteins that are tyrosine-phosphorylated will stick to the antibody column. You can then just release these proteins from the antibody column, collect what you released and then sequence it to identify what those proteins are.
- We can make specific antibodies to phosphorylated tyrosine residues in receptor tyrosine kinases that will bind specific SH2 domain proteins.
- In this case, we are looking at a phosphorylated tyrosine in the context of specific downstream amino acids. You can then use antibodies that recognize these sites to see which tyrosines are phosphorylated in your receptor tyrosine kinase.

Question: **What are the specific residues in a given protein that become phosphorylated?**

- Perform a **triptic protein digest** – you start with your protein that is radioactively labeled, you cut the protein into little pieces with specific proteases: such as Trypsin; cleaves after lysine or arginine residues
- **Protein Kinase C** – serine/threonine kinase. Activated downstream from G-protein coupled receptors that activate phospholipase or for receptor tyrosine kinases that activate phospholipase.



- Separate small fragments on thin layer chromatography gel (dependent on size) or on a polyacrylamide gel, on which you can only see radioactive peptides.

  These are all members of the protein Kinase C family – just showing us that there is one threonine or serine residue that is conserved in all of the members – this is the threonine or serine residue that is present in the activation loop
- We do not know the sequence, but we have two radioactively labeled peptides – we have to identify which peptides these correspond to (at this stage, we don't know where these are localized in the protein)
- **Edman degradation**: you hydrolyze AA by AA by AA; as they are released, you know that you have released certain AA until you come to your phosphorylated residue
- Nowadays, you would use a mass spectrometer – to obtain the mass of that peptide. You could also obtain the sequence of the peptide. This would provide a mechanism to identify the specific residue in that peptide that is phosphorylated.
  - You can treat the cells bydifferent types of activators of this enzyme and you can examine which of those sites are phosphorylated under different conditions, for example.
  - As you purify your peptides by mass spec., what you are looking for is a change in mass that would be predicted if you add a phosphate.

Below: mass spec. of a peptide with one phosphorylated residue

Table 1. Summary of mass-spectrometric analysis of phosphorylation sites of native and phosphatase-treated protein kinase C βII.

Peak	Treatment	Mass[†] (predicted)	Sequence[‡]
1	–	1677.8 (1597.8)	^{636}HPPVL**T***PPDQEVI^{649}R*
	PP1	1597.4	^{636}HPPVL**T**PPDQEVI^{649}R*
	PP2A	1679.7	^{636}HPPVL**T***PPDQEVI^{649}R
2	–	2504.1 (2418.6)	^{503}**T***FCGTPDYIAPEIIAYQPYG^{520}K
	PP1	2422.7	^{503}**T**FCGT/... ^{520}K
	PP2A	2429.2	^{503}**T**FCGT/... ^{520}K
3	–	2771.2 (2690.9)	^{650}NIDQSEFEGFS*FV/... ^{672}K
	PP1	1639.7	^{650}NIDQSEFEGFS FV^{663}K
	PP2A	1638.8	^{650}NIDQSEFEGFS FV^{663}K

[†]Masses were determined by laser desorption mass spectroscopy; underlined masses were determined by ES-MS. [‡]Underlined amino-acid residues were not detected by Edman degradation. Phosphorylated residues are indicated by an asterisk. A backslash denotes where sequencing was terminated. *Sequenced by tandem mass spectrometry.

- Would have a predicted mass of 1597.8
- Mass spec reads 1677.8 – an addition of 80 daltons, which is about the size of the phosphate!
- You can prove whether a phosphate was added or not by the use of a **phosphatase**
- **PP1** is a serine/threonine phosphatase; if you treat this peptide first in the presence of PP1, what happens is that you remove the phosphate and you convert the mass to 1597.4 – shows that this peptide is phosphorylate and that we released the phosphate
- **PP2A**, another phosphatase, fails to dephosphorylate that peptide (not all the phosphatases will dephosphorylate all of the peptides)
- If we look at the second peptide (monophosphorylated), it is dephosphorylatd by both PP1 and PP2A

03/30/2007

Lecture #31–Protein Phosphorylation II

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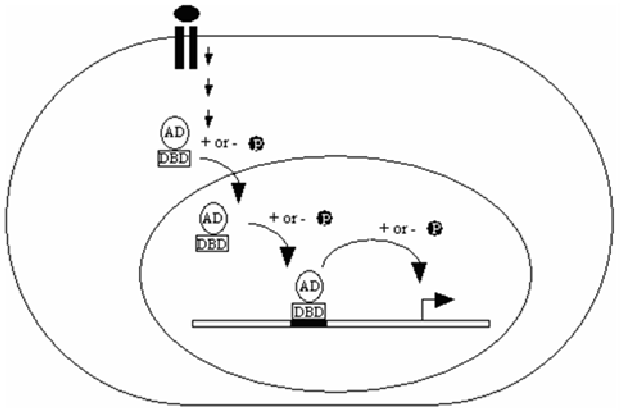
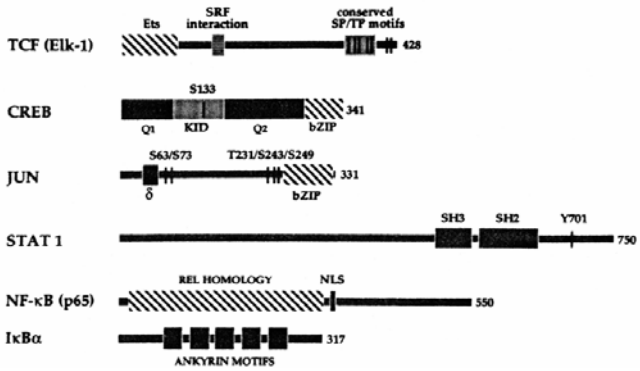
Regulation of Transcription by Phosphorylation:

This diagram shows the different types of processes that could be regulated by phosphorylation.



- A transcription factor may be held in the cytoplasm and cannot get into the nucleus unless it has been phosphorylated; **phosphorylation is regulating the entry or release of factor allowing it to enter into the nucleus**.
- Transcription could be present in the nucleus, but it is not able to bind to DNA; **phosphorylation of the transcription factor regulates it's ability to bind DNA**.
- Transcription factor is bound to DNA through a DNA-binding domain, but it is unable to interact with the transcription machinery and will not activate transcription; **phosphorylation of the transcription factor allows it to interact with the transcription machinery and regulate gene expression**. In this case the transcription factor is negatively regulating gene expression.

Transcription Factors as Targets in Signaling Pathways:



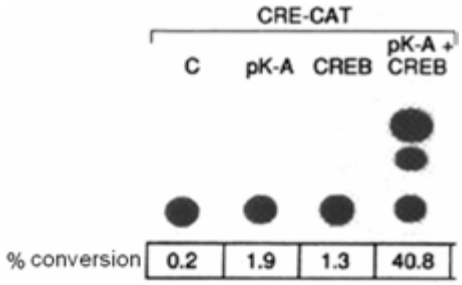
The diagram represents some of the common transcription factors and their DNA-binding domains (which are high-lighted by the hatched boxes). The stripes indicate sites of phosphorylation and some of these proteins are phosphorylated at multiple sites. Example, Jun, has at least 5 different sites of phosphorylation that have been mapped by different kinases. The phosphorylation sites are also in two different domains of the protein. Another example is the TCF (Elk-1) has two phosphorylation sites at the C-terminus. Stat1 contains an SH2 domain (which recognizes phosphorylated tyrosine residues, in the context of a consensus sequence); the SH2 domain allows the recruitment of Stat1 to a signaling complex and once it is recruited the Stat1 transcription factor becomes phosphorylated on a tyrosine residue allowing the dimerization between two Stat1 proteins (through SH2 domains). This dimerization is regulated through phosphorylation of the individual Stat1 molecules. This transcription factor is directly shuttled from the cytoplasm into the nucleus upon phosphorylation; note that in this context the phosphorylation of the tyrosine residue does not have anything to do with the DNA-binding ability of Stat1.

Experimental Approaches in Identifying Regulation of Phosphorylation:

Protein Kinase A and CRE-Binding Proteins:

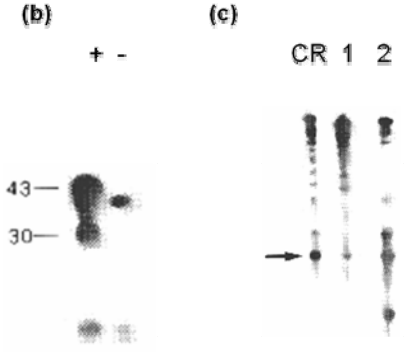
- This occurs downstream of a G-protein coupled receptor that activates **adenylate cyclase** which causes an **induction of cAMP**, which binds to a **regulatory subunit** of **protein kinase A** (also known as **cAMP-dependent kinase**).
- Protein Kinase A is a **serine-threonine kinase**; it remains in the cytoplasm when it is inactive. When cAMP binds the regulatory subunit it releases the regulatory subunit, activating the kinase.
- The active kinase enters the nucleus and phosphorylates a transcription factor known as **CRE Binding protein** (**CREB**) which is bound to DNA at the **cAMP response element** (**CRE**).
- Genes can be regulated by cAMP induction and by following cAMP binding scientists were lead to the downstream kinases.

Scientists have studies genes whose transcription is induced by cAMP levels; they then hypothesized that this may be regulated by protein kinase A. To test this hypothesis, so by knowing genes that are induced the scientists mapped the upstream promoter region and identified a region in the promoter that was required for the induction of the cAMP responsive genes (CRE). CREB is bound to DNA through a DNA-binding domain, but will not activate transcription until protein kinase A enters the nucleus and phosphorylates the activation domain of CREB. The phosphorylation of the activation domain increases the affinity of CREB for **p300** (also known as **CBP** – cAMP binding protein **b**inding **p**rotein). The phosphorylation of the activation domain of CREB causes a conformational change and increases it's affinity for p300/CBP, which is a **histone acetylase**. By allowing CREB to bind p300/CBP, it will acetylate histones and change the conformation of the chromatin in the vicinity of the promoter where CREB is bound; p300/CBP also **recruits the general transcription machinery** (**TFIIB**). p300/CBP can also bind to another histone acetylase, **PCAF**.



Effect of CREB and Kinase A on CRE-CAT Expression:

- Scientists had to do a few experiments to reach this conclusion.
- The scientists had gotten to a stage where they knew there were CRE-Binding elements (which are required domains for transcription activation by cAMP-dependent genes). They had to ask the question: ***what was regulated by phosphorylation?***
- In this experiment the CRE-binding element is upstream from a CAT reporter in a CRE-CAT construct.
- The assay:
  - *Lane 1:* Control
  - *Lane 2:* pK-A alone → this does not activate transcription.
  - *Lane 3:* CREB alone → this does not activate transcription.
  - *Lane 4:* pK-A and CREB → transcription is activated.
- Therefore the CRE-CAT assay shows that both pK-A and CREB are required to activate transcription regulated by cAMP.



Phosphorylation of CREB Protein *in vitro* and *in vivo:*

- In this case the scientists purified proteins in the cell that can bind to the CRE element through **affinity chromatography**.
- So you have a column with the CRE element and you purify proteins that can bind that element in the absence of additional phosphorylation.
- Then the proteins were eluted from the affinity column and pK-A and ^{32}P of γ-labeled ATP were added.
- Then pK-A will phosphorylate the proteins that were eluted from the column chromatography.

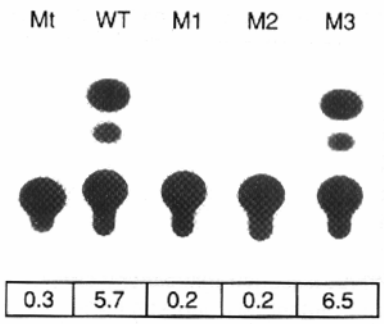
(b) In the control lane (-) there is no pK-A, only the eluted protein and the labeled ^{32}P/ATP, just in case there may be a kinase bound to the column. This shows that in the absence of pK-A there is some phosphorylation of a protein, but when pK-A is added there is a very high level of phosphorylation of two specific proteins. The first protein is present in the upper-band (43 kDa protein), which corresponds to CREB. This can be used to map what sites on CREB are phosphorylated by pK-A.

(c) Is an *in vivo* experiment where the cells have been labeled with $^{32}P_i$, this will label everything in the cell (all proteins that are phosphorylated and DNA/RNA molecules). CR represents the control where the CREB runs alone. Lane 1 is the ethanol control and lane 2 the cells are stimulated with forskolin (which induces levels of cAMP). Generally you see a specific increase in a protein that runs at the same size as CREB; other proteins are increased as well, but these proteins are purified through affinity chromatograph with CRE as the stationary phase. This tells you the proteins can bind in the absence of phosphorylation and be phosphorylated afterwards, it also tells you that cAMP levels are required for phosphorylation of the target proteins.

Characterization of CREB Point Mutants:

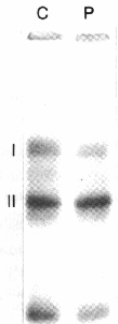
- Now we want to know :
  - *What sites are important to regulate this event?*
  - *What events are phosphorylated by pK-A?*
  - *Are these required for transactivation of transcription?*
- By mapping what sites can be phosphorylated, scientists had identified a **serine residue** that could be phosphorylated by either pK-A or another serine/threonine kinase called pK-C. They knew that pK-A required amino acids upstream for efficient phosphorylation of the serine and pK-C required amino acids downstream for efficient phosphorylation of the serine.
- So they made a number of mutants by substituting the serine with alanine/asparagines (to disrupt phosphorylation) or they substituted amino acids downstream, such that phosphorylation of the serine by pK-C would be blocked but pK-A would still be able to phosphorylate.
- The CAT assays were performed with the mutants and they looked for the ability of pK-A to induce transcription from the CRE-CAT construct.

Construct	(130) pK-A	pK-C (136)
Wild Type	. . . .AGG AGG CCT TCC	TAC AGG AAA. . . .
	. . . .Arg Arg Pro Ser*	Tyr Arg Lys. . . .
M1	. . . .AGG AGG CCT GCC	TAC AGG AAA. . . .
	. . . .Arg Arg Pro **Ala**	Tyr Arg Lys. . . .
M2	. . . .AGG AGG CCT GAC	TAC AGA AAA. . . .
	. . . .Arg Arg Pro **Asp**	Tyr Arg Lys. . . .
M3	. . . .AGG AGG CCT TCC	TAC ATG GAA. . . .
	. . . .Arg Arg Pro Ser*	Tyr **Met Glu**. . . .



The wildtype showed good induction of transcription. The first mutant (substituted alanine) gave rise to no transcription. Same for mutant 2 (substituted with asparagines). Mutant 3 showed transcription, which meant that the protein was activated by pK-A (since pK-C cannot phosphorylate due to the altered consensus sequence). This tells us that this site is essential to activate transcription and in this context pK-A will activate it.

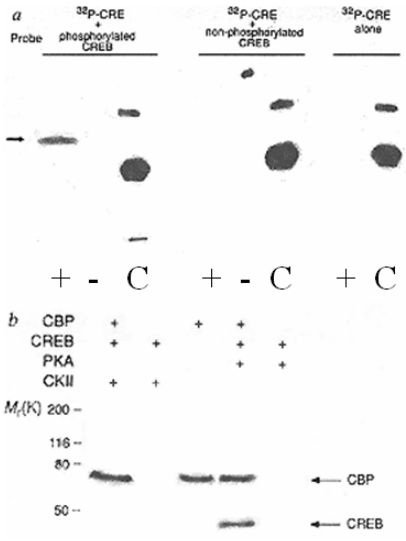
Now we know that the serine residue must be phosphorylated to activate transcription, we know that if we stimulate cells with forskolin (increasing cAMP) phosphorylation of CREB will be enhanced. We know that CREB can bind DNA in the absence of phosphorylation, but when the proteins are eluted we can phosphorylate the protein (and use that to map the site). The next question would be: ***does CREB need to be phosphorylated to bind DNA?***



- Scientists made CREB in bacteria and observed its capacity to bind the cAMP response element *in vitro*. In 'C' they took proteins that were not phosphorylated and compared to 'P' where proteins were phosphorylated *in vitro* at the serine residue. The results showed that there was no difference in the capacity of CREB to bind DNA when comparing the phosphorylated and unphosphorylated

states. This provides evidence that this protein can bind DNA in the non-phosphorylated state and it is previously know that CREB must be phosphorylated to activate transcription. **Therefore it is clear that phosphorylation only regulates transcription not DNA-protein binding.**

- The scientists had been able to purify complexes containing CREB, and it was known that CREB can form a complex with p300/CBP. The next question is: ***does phosphorylation regulate the ability of p300/CBP to bind CREB?***



The diagram represents another association assay.

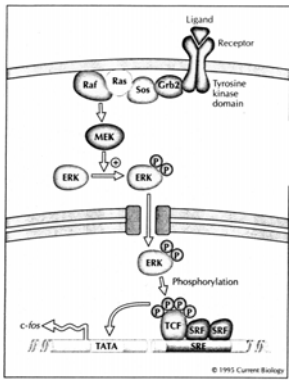
- This assay was performed with labeled-CRE which is bound to CREB and the assay is run with phosphorylated and unphosphorylated CREB.
- The CREB samples are bound to the protein binding domain of p300/CBP and CREB only binds p300/CBP when it is phosphorylated. Non-phosphorylated CREB does not bind to p300 and ^{32}P-CRE also does not bind to p300/CBP.
- **Therefore, CREB cannot bind p300/CBP until it is phosphorylated**. When CREB is phosphorylated it can transcativate transcription.
- Therefore, the next step is to co-immunoprecipitate p300/CBP and CREB. In this experiment we are asking: ***is phosphorylation of CREB required for co-IP?*** In the presence of pK-A, CREB, p300/CBP and ^{32}P we can see CREB and p300/CBP coimmunoprecipitate; CREB is phosphorylated with pK-A.
- In the next lane we want to see if CREB is phosphorylated by another kinase (CKII). p300/CBP will still immunoprecipitate but CREB will not co-IP; CKII will not phosphorylate CREB. Therefore, phosphorylation of CREB by pK-A is required to promote a binding interface with CBP.

Conclusion:

Now we know that phosphorylation of CREB is required for transactivation of transcription and now we know that CREB can bind DNA in the absence of phosphorylation. We also have mapped the site of phosphorylation by pK-A (purification of the radiolabeled CREB) on the serine residue. We've demonstrated that CREB must be phosphorylated with pK-A and that CKII cannot activate transcription. We have shown that once CREB is phosphorylated it can interact with p300/CBP and that pK-A phosphorylation of CREB, and not CKII phosphorylation of CREB, is required to generate this complex.

Mechanism of Short-Term and Long-Term Memory:

Since cAMP is a short-lived second messenger, scientists then asked: ***what is the consequence of the duration of the signal (example: cAMP)?*** Scientists looked at the behaviour of slugs and they showed that if cAMP release was stimulated for a short period of time pK-A phosphorylated proteins present in the cytoplasm. If cAMP was activated for a prolonged period of time, this released higher levels of pK-A which entered the nucleus and phosphorylate CREB and induce a series of gene known as **immediate-early genes** (**IEGs**). These IEGs stimulate synthesis of neurotransmitter enzymes and neuroendorphin receptors that were required for the slug to respond to the signal. The transient response did not induce the same response as the prolonged response.

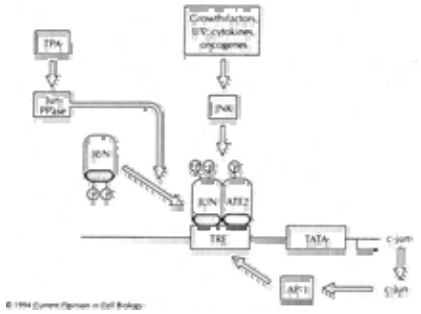


Activation in the Nucleus by MAP Kinase Pathways:

Ras Pathway:

An example of this is the **Ras pathway**, which activates a series of serine/threonine kinases (Raf, MAP Kinasae, MAPK Kinase). The endpoint of all of these signaling pathways are called **MAP kinases** (**mitrogen activated kinases**), and they can all enter the nucleus and phosphorylate transcription factors. Once the RTK is activated it will activate the MAP kinase (in the case of the Ras the MAP Kinase is called ERK → serine/threonine kinase) which enters the nucleus and activates the **serum response**

element (**SRE**) this is a historical name. ERK enters the nucleus and phosphorylates TCF at a number of sites, and this can recruit the basal transcription machinery and activate transcription of genes that have this upstream binding element. This can occur within 30 minutes of stimulation, yields synthesis of another TF called FOS.

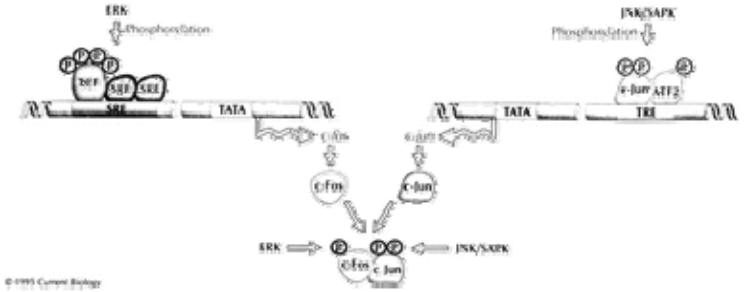


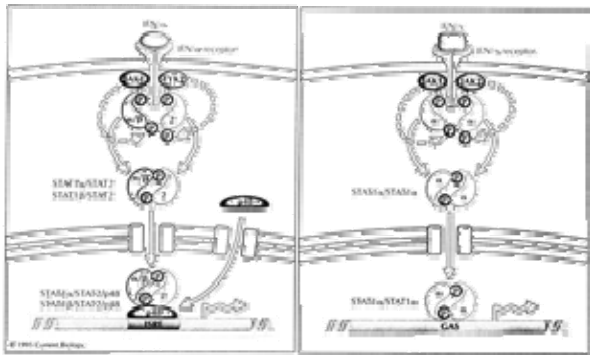
Jun Pathway:

Jun kinase (JNK) phosphorylates a transcription factor called Jun, which is serine/threonine kinase which is activated by growth factors. Jun enters the nucleus and phosphorylates the Jun transcription factor and ATF2, already bound to DNA at TRE elements which are upstream of the *Jun* gene. Jun can also be phosphorylated at its DNA binding domain, which causes Jun to lose affinity for the TRE, and even in the presence of Jun kinase it cannot activate transcription.

AP-1 Activity:

The endpoint of activating ERK MAP Kinase is the production of transcription factor, called **Fos**, and Jun MAP Kinase produces more Jun transcription factor. Therefore the cellular levels of Fos and Jun are high and they form a complex, called **AP-1**, which binds DNA and transactivates another set of genes. AP-1 is required to activate the transcription of genes that are required to get through the G_1 phase of the cell cycle up until the G_1/S-checkpoint.





JAK-STAT Mechanism:

These transcription factors contain and SH2 domain and are phosphorylated on tyrosine residues. There are several STAT factors (such as STAT1 and STAT2), they are recruited when the cytokine receptor is phosphorylated. When the cytokine receptor binds its ligand, it recruits tyrosine kinases. The receptor then gets phosphorylated on tyrosine residues, and the SH2 domain will bind to the phosphotyrosine residue and then it's recruited to the receptor complex. In this complex STAT becomes phosphorylated on tyrosine residues. The tyrosine on STAT has a higher affinity for the SH2 domain of another STAT, and thus STAT forms a dimeric complex of STAT1-STAT1 or STAT1-STAT2. This causes a conformational change which exposes a **nuclear entry signal** in the STAT factor. Then this dimer enters the nucleus and binds to DNA (either directly to GAS elements or indirectly through additional binding proteins already bound to DNA). In this context these transcription factors are **not** phosphorylated in the nucleus.

NFκB and Dorsal:

These are other types of receptors, NFκB is present in mammalian cells and Dorsal arises in Drosophila. These pathways both activate cytokine receptors which will activate a serine/threonine kinase. In mammalian cells this kinase is called **κB kinase**. This will phosphorylate a transcription factor (2 subunits) known as NFκB, which is held in the cytoplasm held to an inhibitory protein (Inhibitor of Kappa B - **IκB**). κB kinase phosphorylates the inhibitor and releases NFκB and targets the phosphorylated inhibitor protein for degradation.