Growth Factors and Receptor Tyrosine Kinases

- RTK’s—How do they work?
- EGFR signaling and ras
- MAP kinase cascades
- PI3K, PKB, PLCγ
- PTPs (Protein Tyrosine Phosphatases)

Epidermal growth factor

Neurotrophic growth factor (NGF) isolated from mouse submaxillary glands (Rita Levi-Montalcini)

Stanley Cohen, 1962

"Side effects" of impure NGF preps
- Premature eyelid opening (7d vs. 14 d)
- Premature tooth eruption (6 d vs. 9 d)
- Pure "Tooth-lid factor" = EGF

Important roles in development
- Mitogenic for fibroblasts
- Regulates growth/differentiation of many target cells

Refs:
- S. Cohen, Nobel lecture, 1986

Phospho-tyrosine signals

Kinases phosphorylate tyrosine (Y*) residues of target proteins

Y~P = target for distinctive protein binding pockets, with surrounding sequences lending specificity

ALWAYS activate by promoting proximity of proteins A and B
(sometimes by allostery also)

In its new proximity to A, B’s activity (= X) can now:
- Phosphorylate or de-phosphorylate another protein
- Make or degrade a 2nd messenger
- Attract additional signaling molecules

Y~P provides long-lasting but erasable memory, which is terminated by DE-phosphorylation

*Y = one-letter code for tyrosine; S = ser, T = thr, etc.
Phospho-tyrosine signals regulate growth & differentiation

RTKs = Receptor Tyrosine Kinases

Extracellular region variable, with many different motifs
Usually cross membrane only once
Intracellular region contains conserved catalytic domains

ALSO: TK-linked receptors for:
- Antigens (receptors on B and T cells)
- Growth hormone
- Interleukin-4
- Erythropoietin, many others

How RTKs (& TK-linked Rs) work

1. Ligand promotes formation of RTK dimers, by different mechanisms:
   - Ligand itself is a dimer (PDGF)
   - One ligand binds both monomers (GH)

2. Dimerization allows trans-phosphorylation of catalytic domains, which induces activation of catalytic (Y-kinase) activity

3. Activated TK domains phosphorylate each other and proteins nearby, sometimes on multiple tyrosines

4. Y~P residues recruit other signaling proteins, generate multiple signals

EGF receptor as a model

1st RTK to be characterized
v-erbB oncogene = truncated EGFR

Evidence for EGFR dimerization

Yarden & Schlessinger

Rate of phosphorylation = \( k[\text{EGFR}]^2 \), even in micelles!
Therefore: 2 EGFRs required for phosphorylation

Later confirmed by

- Chemical cross-linking
- FRET
- Dominant-negative mutants (e.g., kinase-dead EGFR)

IMPORTANT

Dimerization/proximity = alternative to allostery
(Shown by swapping EC/IC domains of EGFR, PDGFR)
How do we know that the EGFR auto-phosphorylates *in trans*?

Experiment: test WT and short EGFRs, each with or without a kin’ mutation

Honneger et al. (in vitro) PNAS 1989; (in vivo) MCB 1999

Does this result rule out phosphorylation *in cis* as well?

If not, how can you find out?

PS: What do *trans* and *cis* mean?

How can we know that the EGFR does *not* autophosphorylate *in cis*?

- Need an EGFR that cannot homodimerize
- EGFR family is huge, with many RTK members and many EGF-like ligands
- Such receptors often form obligatory heterodimers with a similar but different partner
- If A can dimerize only with A’, then we can inactivate the kinase domain of A’ and ask whether A phosphorylates itself

Answer: NO

QED

How does dimerization activate RTKs?

GFRTs (like many kinases) have sites in their T loops at which phosphorylation activates

Dimerization induces T-loop phosphorylation *in trans*

Phosphorylation of Y (one or more) in T-loop causes it to move out of the way of the active site.

Proximity by itself is usually enough to promote T-loop phosphorylation, but there may also be a role for allostery

Once activated, each monomer can phosphorylate nearby Y residues in the other, as well as in other proteins
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Signals generated by the EGFR
The activated dimer phosphorylates itself
Individual Y-P residues recruit specific proteins, generate different signals
SOS, a Ras GEF
  Docks via intermediate adapters to activate Ras
  Ras activates multiple targets (MAPK)
P.LCγ
  Docking of Y-kinases allows Tyr-phos’n of PLCγ, which activates it
PI3-kinase
  Adapters again
  Docking allosterically activates PI3K
Each signal, in turn, activates a different set of pathways, which cooperate to produce the overall response

Adapters connect A with B, B with C . . . to create complex localized assemblies of signaling proteins
Each adapter has at least 2 interaction domains, and may have other functions as well

Types of adapter interactions
Y-P sequence motifs allow regulatable adapter functions
  SH2 Tyrosine phosphates
  PTB Tyrosine phosphates
Also
  SH3 Polyproline-containing sequences
  PDZ Specific 4-residue sequences at C-termini
  Pleckstrin homol. (PH) Phosphoinositides
  Many others
SH2 & SH3 domains--src homology domains

SH domains are protein domains initially discovered in Src, a transforming tyrosine kinase found in Rous sarcoma virus.

Sequences of many signaling proteins that interact with RTKs revealed multiple homologous domains to Src region 2 and region 3.

SH2: Protein motif of ~100 amino acids, binds to phosphotyrosine peptide sequences. (87 SH2 in the human genome)

SH3: ~60 amino acid domain, binds to R-X-X-P-X-X-P peptide sequences. (143 SH3 in the human genome)

How would you determine the specificity of an individual SH2 domain for a phosphopeptide?

EGF activates the MAPK pathway in multiple steps with multiple mechanisms

Fly genetics to the rescue

Fly eye consists of ~800 ommatidia, an individual lens structure consisting of 22 cells (8 photoreceptor cells, R1-R8)

Eye development is a highly ordered process. RTK signaling is essential. Mutation in sevenless results in loss of R7. Additional mutations in pathway identified sos (son-of-sevenless), boss (bride of sevenless), Drk (downstream of receptor kinase)
EGFR Activation of Ras: Proximity & Allostery

The Players

RTK = EGFR

“Rat Sarcoma” Small GTPase, attached to PM by prenyl group

“Son of Sevenless” GEF, converts Ras-GDP to Ras-GTP
Found in Drosophila homol. To S.c. Cdc25

SH2 SH3 Grb2 SOS

EGFR Activation of Ras: Proximity & Allostery

Even before EGF arrives . . .

SOS is “ready to go”:
already (mostly)
associated with Grb2 in cytoplasm, in the resting state

EGFR Activation of Ras: Proximity & Allostery

Then . . . Covalent modification

EGF-bound dimers trigger phosphorylation, in trans

SH2 Grb2 SOS
Grb2’s SH2 domain binds Y~P on EGFR, bringing SOS to the plasma membrane.

SOS now binds Ras-GDP, causing GDP to dissociate, and...

GTP enters empty pocket on Ras, which dissociates from SOS and converts into its active conformation.
EGFR Activation of Ras: Proximity & Allostery

Finally . . . Proximity again!

Ras-GTP brings Raf to the PM for activation, and the MAPK cascade is initiated.

How does Ras activate Raf? Proximity vs. allostery

Allostery: Ras recruits Raf to the PM and activates it directly

Proximity: Ras recruits Raf to the PM, where it is activated by X

How can we tell the difference?

Does Raf signal (without Ras) when recruited to the PM?

Experiment
Attach a CAAX* box to Raf’s C-terminus
Express Raf-CAAx in cells, measure activity of MEK, an enzyme downstream in the MAPK pathway

Answer: “proximity +”

Ras does localize Raf but does not activate it (other proteins do)

Stokoe et al. (1994) Science

EXV
Raf
Raf/RasG12
Raf/CAAX
Raf/CAAX/RasG12

Relative MEK activity

*CAAX (A = aliphatic; C = cysteine) is a site for prenylation; prenylated proteins concentrate at the PM
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The best understood MAPK cascade

MAPK = Mitogen-activated protein kinase

The diagram illustrates the MAPK cascade, showing the activation of different kinases and the phosphorylation of various substrates. The cascade includes Raf-1, MEK1, MEK2, ERK1, and ERK2, with the final effect being the phosphorylation of c-Jun and alterations in gene expression.

The diagram is borrowed from Chan, STKE.
MAPK “cassettes” mediate many different responses

Vertebrates  Frog oocyte  S. cerevisiae
Mitogens  Progesterone  Mating pheromone
MAPKKK  MAPKK  MAPK
MAPK

Different biology, similar cassettes: why 3 kinases?
- Additional sites for regulation
- Combinatorial diversity
- Magnitude amplification
- Switch-like responses

Switch-like behavior*

Responses are not always graded


All or nothing response in Xenopus oocytes

Progesterone, or fertilization, induces germinal vesicle breakdown of Xenopus oocytes—a process mediated by the MAPK cascade.

Question: At a concentration of progesterone that half-maximally activates MAPK (0.01 uM, panel A), are all the oocytes activated halfway (panel B), or are half of the oocytes activated fully (panel C)?

Since Xenopus oocytes are HUGE, one can look at MAPK on a cell by cell basis.

Answer: All or nothing.

Of course, life is not so simple . . . BONUS slide

Does this work in mammalian cells?

Blenis and co-workers used FACS and immunohistochemistry (anti-DP ERK Ab) to look at EGF activation of ERK in Swiss 3T3 fibroblasts

MacKeigan MCB 2005

Scaffolds for MAP Kinase signaling

Deletion analysis of the binding of JIP-1 to JNK1, MKK7, MLK3, and DLK. JIP-1 was expressed in cells as a GST fusion protein together with HPK1 or epitope-tagged JNK1, MKK7, MLK3, and DLK (15, 16). The presence of these kinases in glutathione-agarose precipitates was examined by protein immunoblot analysis.

HPK = hematopoietic progenitor kinase
DLK = dual lineage kinase (member of the MLK family)

Whitmarsh et al. (1998) Science 281: 1671

Scaffolding roles of JNK-interacting proteins

Dhanasekaran (2007) Oncogene
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**PI3-K pathway and Cancer Syndromes**

- PI3-K
- GF
- RTK
- PTK
- AKT1/2
- Homartin
- Tuberin
- RheB
- mTOR
- S6K
- 4EBP-1
- PIP3
- Tyrosine Kinase
- Ser/Thr Kinase
- GEFs
- Small GTPase
- Inhibitor of eIF4E
- IP3 receptor
- Protein synthesis
- Cell growth/size/survival

**Tuberous Sclerosis Complex**

- TSC1
- TSC2
- Tuberin
- Hamartin

**PI3-K pathway and Cancer Syndromes**

**PI3 targets** include many GEFs, many tyrosine kinases, and others, including . . .

**PKB (aka Akt)** = ser/thr kinase that promotes cell survival

**PKB**

- . . . is inactive in cytoplasm
- . . . contains a PH (pleckstrin homology) domain & a kinase domain

**Multi-step activation of PKB: proximity**

- PH domain recognizes 3'-phosphate of PIP3, bringing kinase domain to the PM
- Proximity to PM alone does not activate the kinase
Multi-step activation of PKB: covalent modification

Inactive PKB → Active (phos’d) PKB

*PDK is also recruited to the membrane via a PIP3-binding PH domain

Overall, two proximity steps plus (at least) one phosphorylation step

EGFR Activation of PLCγ combines THREE inputs

1. PROXIMITY: Recruitment from cytoplasm to PM, via SH2 domains

2. COVALENT: Activated by EGFR phosph’n

3. PROXIMITY: Binds to PIP3 via PH domain

PIP3

EGFR activation combines THREE inputs
Summary: Many RTK effectors require two or more simultaneous inputs for activation

**PI3K**: recruitment via SH2, allostERIC regulation by EGFR/p85

**PKB**: recruitment, phos’n by non-EGFR-kinase(s)

**PLCγ**: recruitment, phos’n, retention at PM by binding PIP3

Why multiple inputs to each effector?

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RTKs activate a complex network of interacting response pathways (and this is the simple version!)

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But how do you shut these things off?
Family of Protein Phosphatases

How Do PTPs dephosphorylate specific targets?

Intracellular targeting: “zip code” model
Extra domains on PTPs confer localization and protein-protein interactions
Initially thought that catalytic domains possessed little specificity for RTKs. However, co-crystal structures and biochemistry reveal that some PTPs catalytic domains exhibit exquisite sensitivity

PTEN opposes PI3K by removing PI3-phosphate

PTEN discovered as a tumor suppressor gene.
Mutated in brain, breast and prostate cancers.
Has homology to dual specificity phosphatases, but shows little activity toward phosphoproteins.
Was discovered to remove phosphates from PIPs; thereby providing likely mechanism for tumor suppression.
Gleevec--proof that you can target kinases for drug therapy

Goldman & Melo, NEJM, Oct 9, 2003