5 CELL SIGNALING

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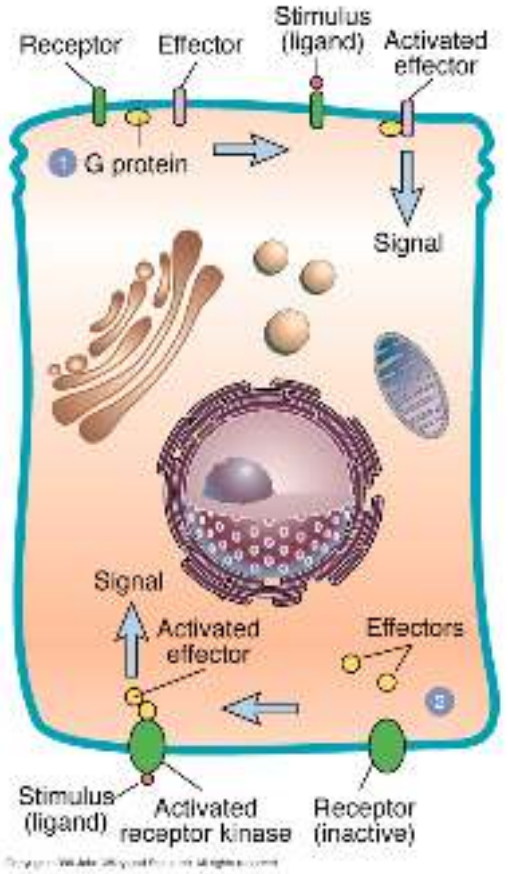


Suggested Reading: Lodish, Chapter 13; Chapter 14, 14.3 to 14.5

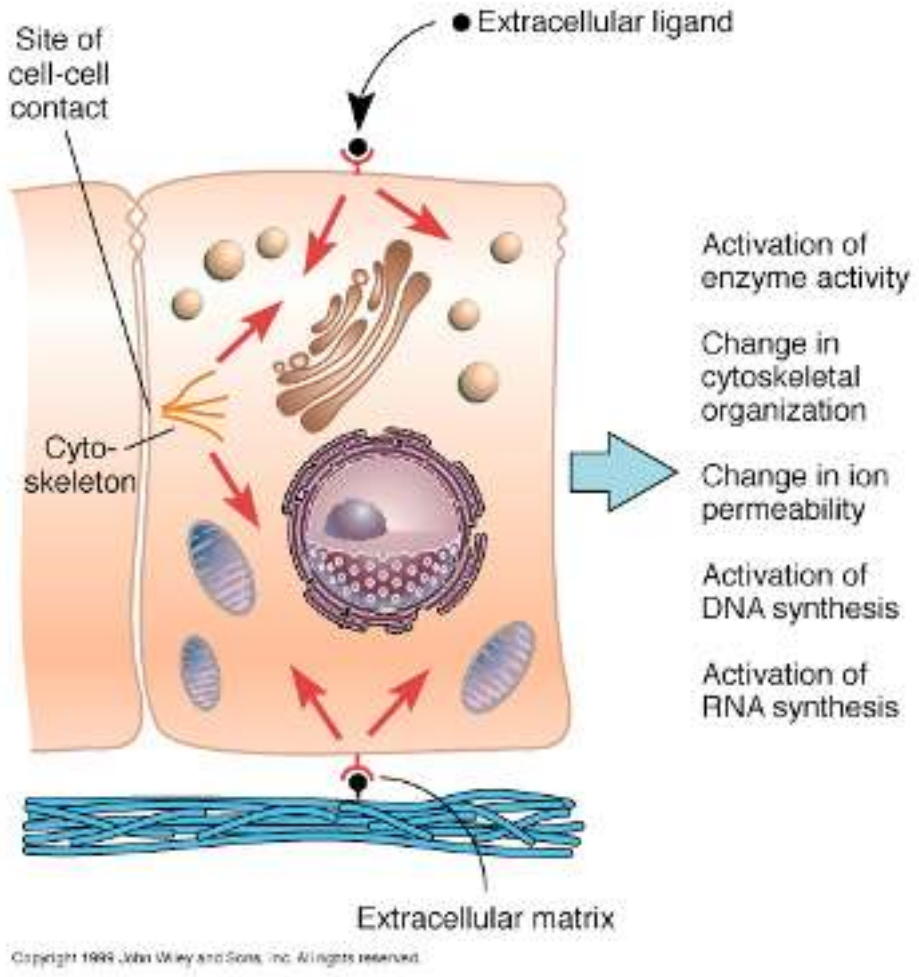
Alberts, Chapter 15

I INTRODUCTION TO SIGNALING PATHWAYS

Essential to the survival of every cell is to monitor the environment and to respond to external stimuli. For most cells this includes appropriate communication with neighboring cells. Cell signaling (or signal transduction) involves:

- Detection of the stimulus (in most cases a molecule secreted by another cell) on the surface of the plasma membrane.
- Transfer of the signal to the cytoplasmic side.
- Transmission of the signal to effector molecules and down a signaling pathway where every protein typically changes the conformation of the next down the path, most commonly by phosphorylation (by kinases) or dephosphorylation (by phosphatases).

The final effect is to trigger a cell's response, such as the activation of gene transcription



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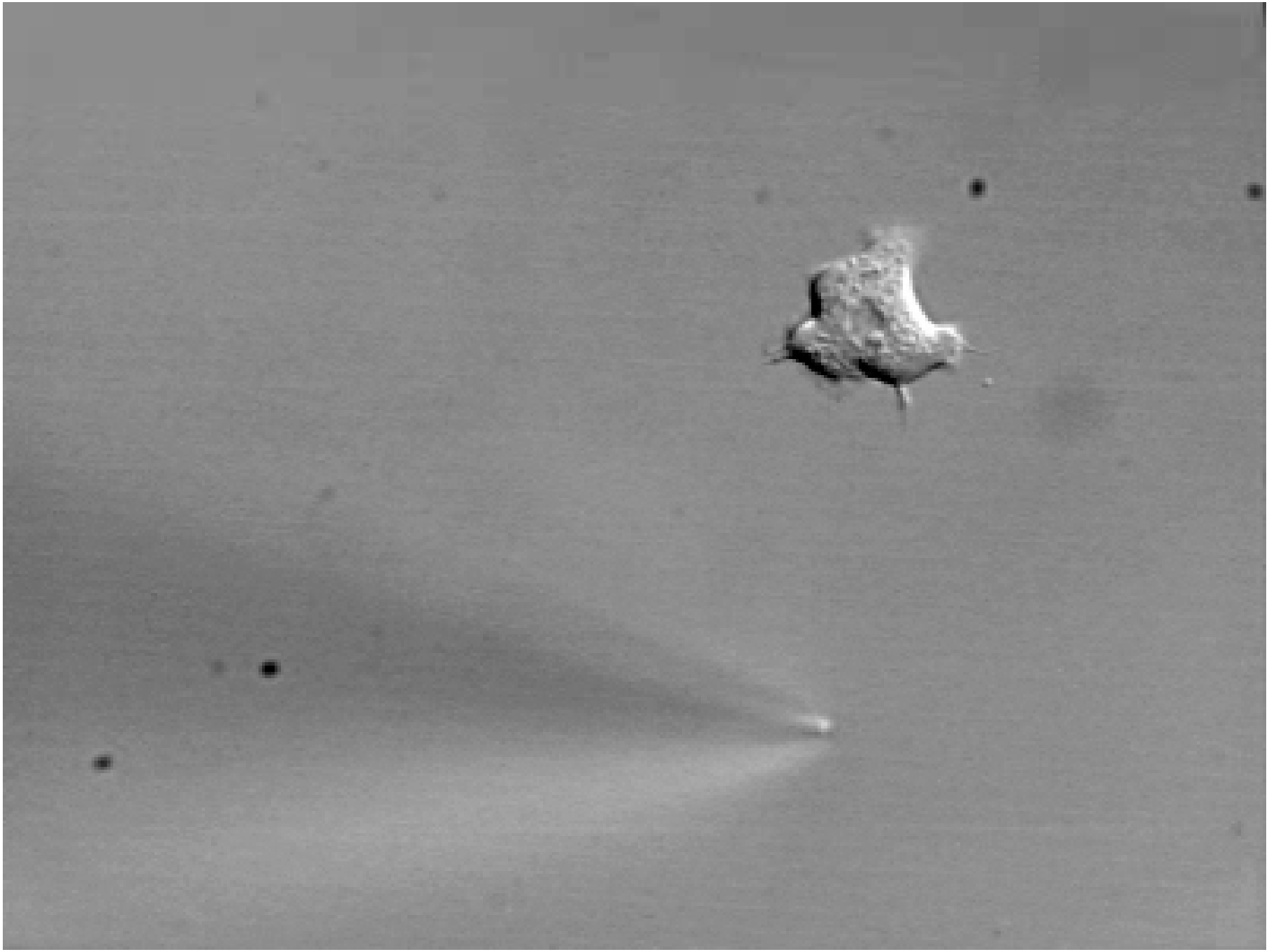
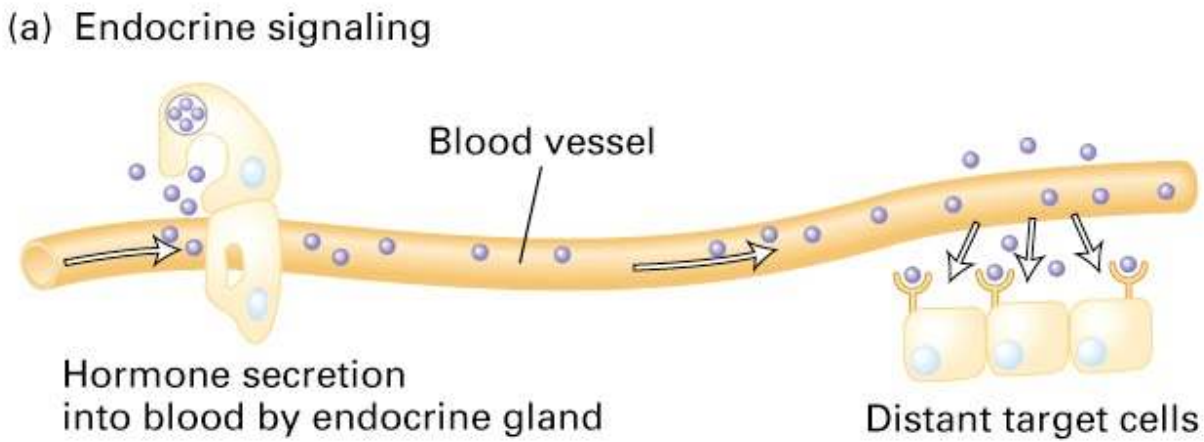


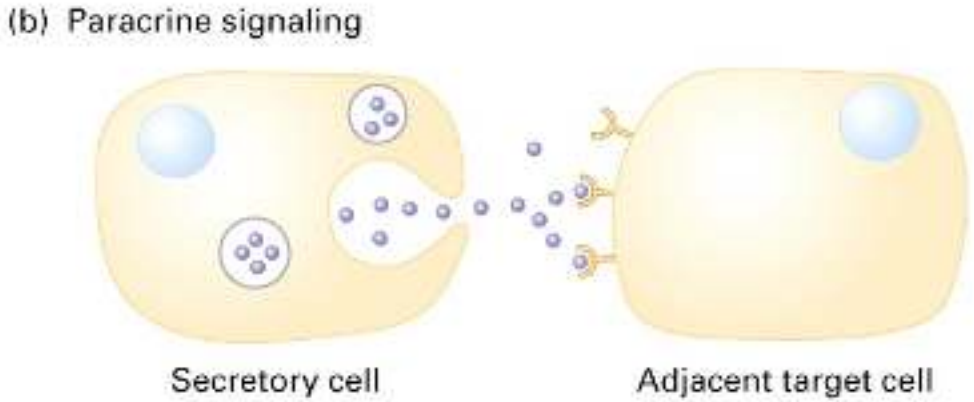
I A – Types of Signaling

Cells communicate by means of extracellular signaling molecules that are produced and released by signaling cells. These molecules recognize and bind to receptors on the surface of target cells where they cause a cellular response by means of a signal transduction pathway.

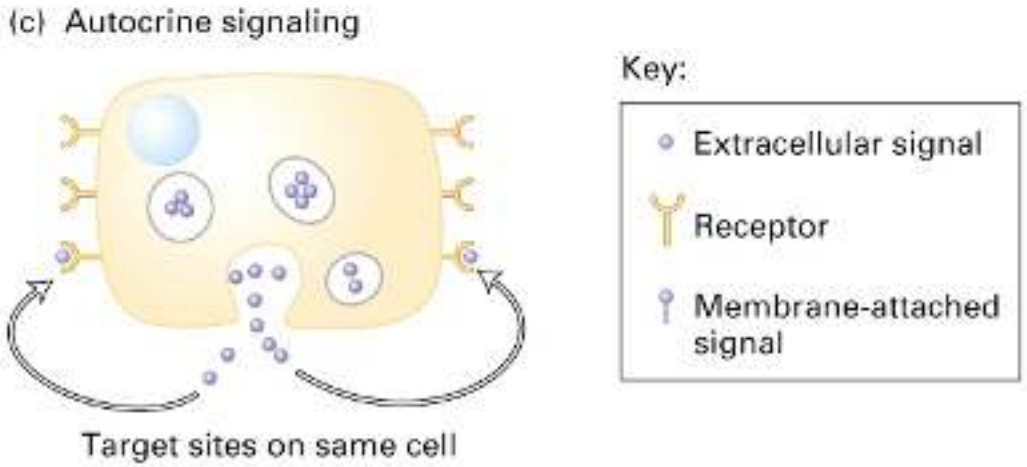
Depending on the distance that the signaling molecule has to travel, we can talk about three types of signaling:



In **endocrine signaling** hormones are produce by an endocrine gland and sent through the blood stream to distant cells.
Hormones can be: small lipophilic molecules that diffuse through the cell membrane to reach cytosolic or nuclear receptors. Examples are progesterone and testosterone, as well as thyroid hormones. They generally regulate transcription; or water soluble molecules that bind to receptors on the plasma membrane. They are either proteins like insulin and glucagons, or small, charged molecules like histamine and epinephrine.



In paracrine signaling the signaling molecule affects only target cells in the proximity of the signaling cell. An example is the conduction of an electric signal from one nerve cell to another or to a muscle cell. In this case the signaling molecule is a neurotransmitter.

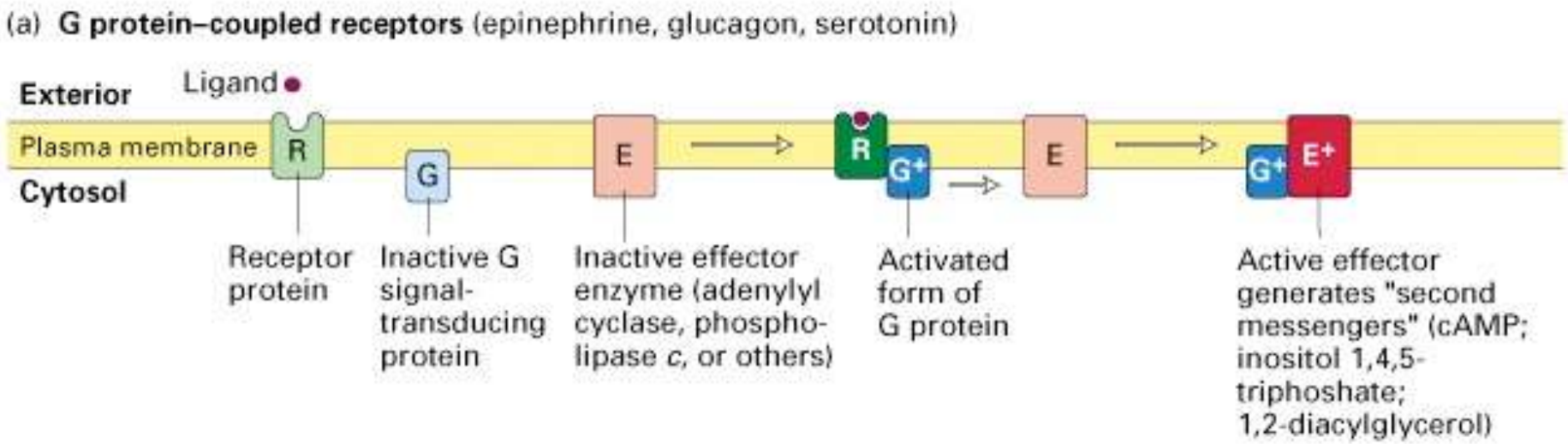


In autocrine signaling cells respond to molecules they produce themselves. Examples include many growth factors. Prostaglandines, lipophilic hormones that bind to membrane receptors, are often used in paracrine and autocrine signaling. They generally modulate the effect of other hormones.

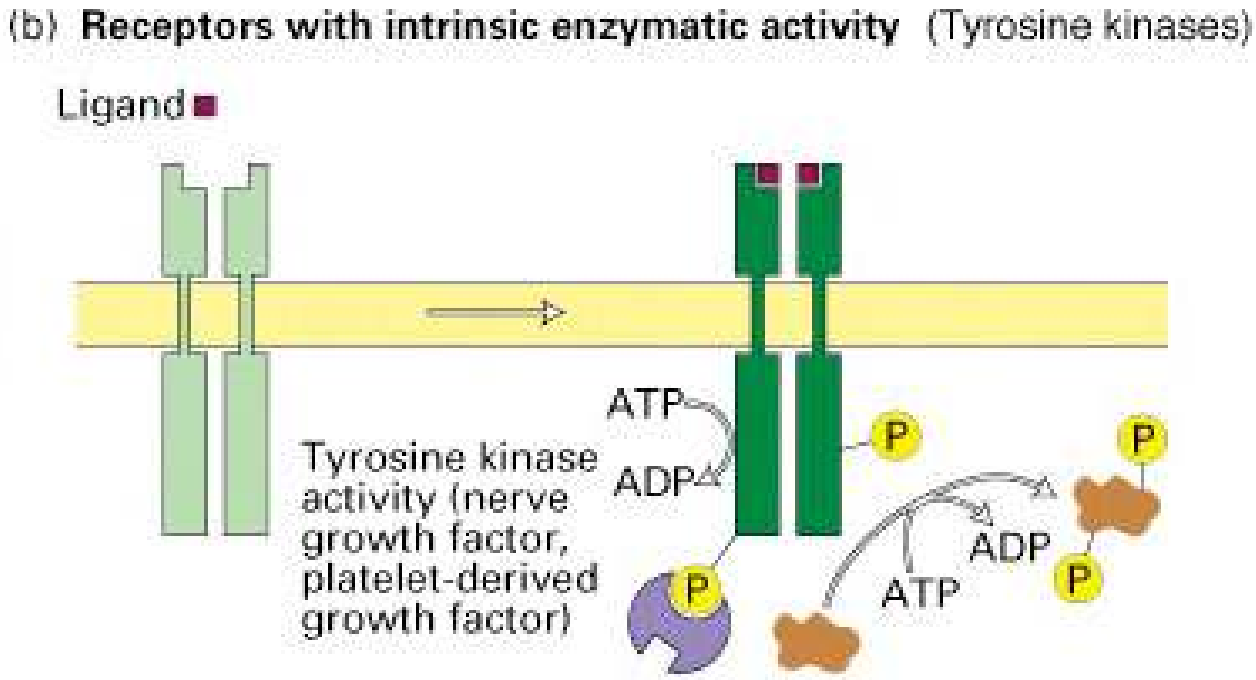
Once a signaling molecule binds to its receptor it causes a conformational change in it that results in a cellular response. The same ligand can bind to different receptors causing different responses (e.g.. acetylcholine). On the other hand, different ligands binding to different receptors can produce the same cellular response (e.g. glucagon, epinephrine).

I B – Types of Receptors

There are a number of receptor classes that are used in different signaling pathways. The two more predominant are:



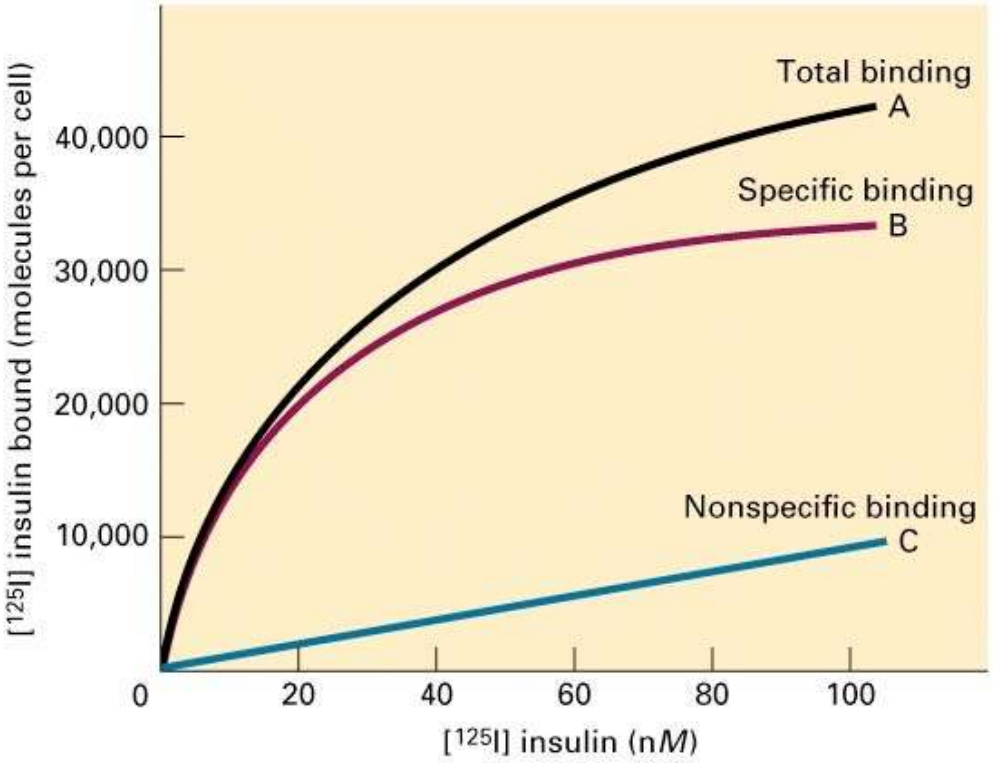
The conformational change in the receptor upon ligand binding activates a G protein, which in turns activates an effector protein that generates a second messenger.



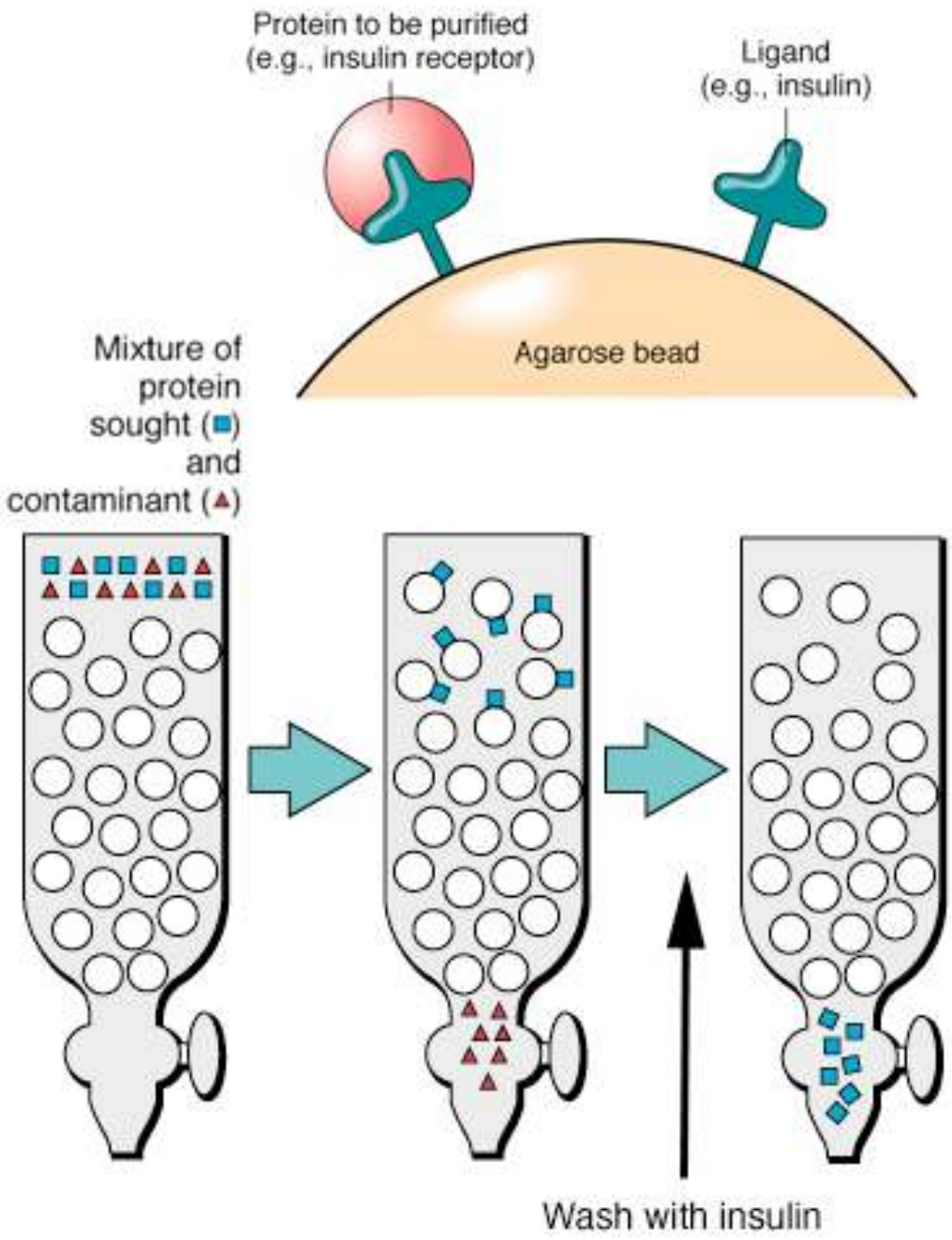
These receptors have a catalytic activity that is activated by binding of the ligand. An example are tyrosine-kinase receptors. Binding of an often dimeric ligand induces dimerization of the receptors that leads to cross phosphorylation of the cytosolic domains and phosphorylation of other proteins.

Identification and Purification of Cell-Surface Receptors

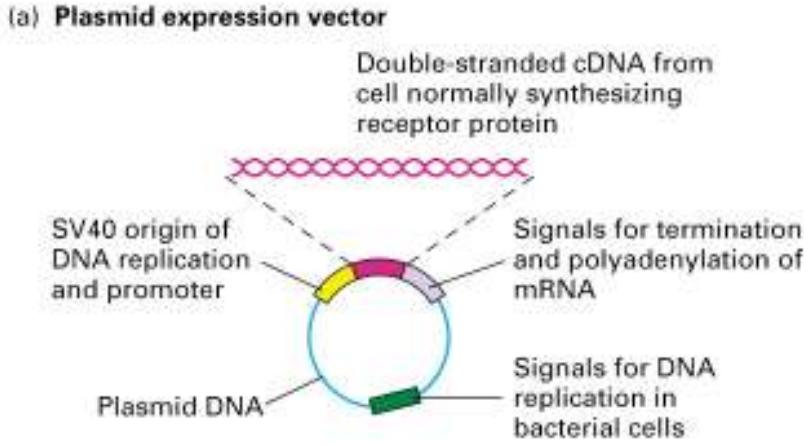
Hormone receptors are difficult to identify and purify because they are present in very low abundance and they have to be solubilized with nonionic detergents. Given their high specificity and high affinity for their ligands, the presence of a certain receptor in a cell can be detected and quantified by their binding to radioactively-labeled hormones. The binding of the hormone to a cell suspensions increases with hormone concentration until it reaches receptor saturation. Specific binding is obtained by measuring both the total and the non-specific binding (which is obtained by using a large excess on unlabeled hormone).

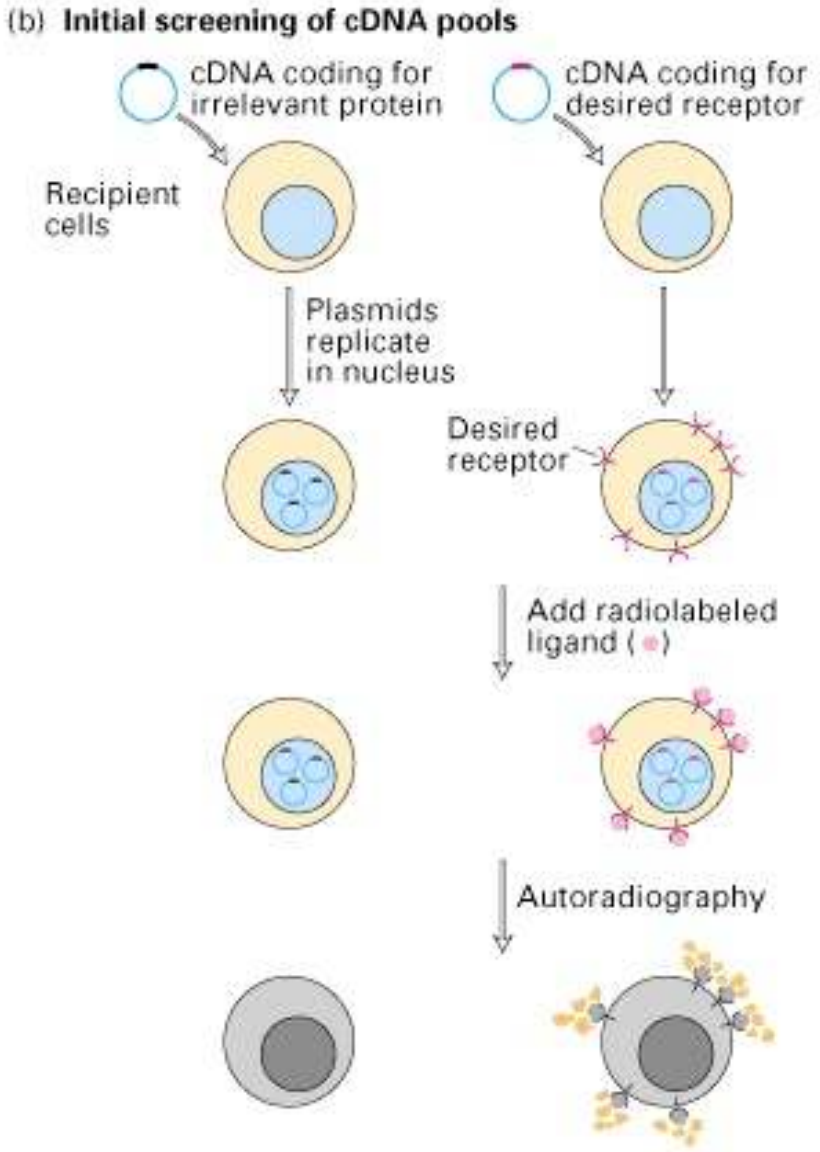


The receptor can be purified in some cases by means of affinity chromatography: the hormone is linked to agarose beads. Crude, solubulized membranes are passed through a column with these beads, which will retain only their specific receptor. The receptor is later release by passing excess hormone through the column. A single pass can produce a 100,00 fold enrichment.

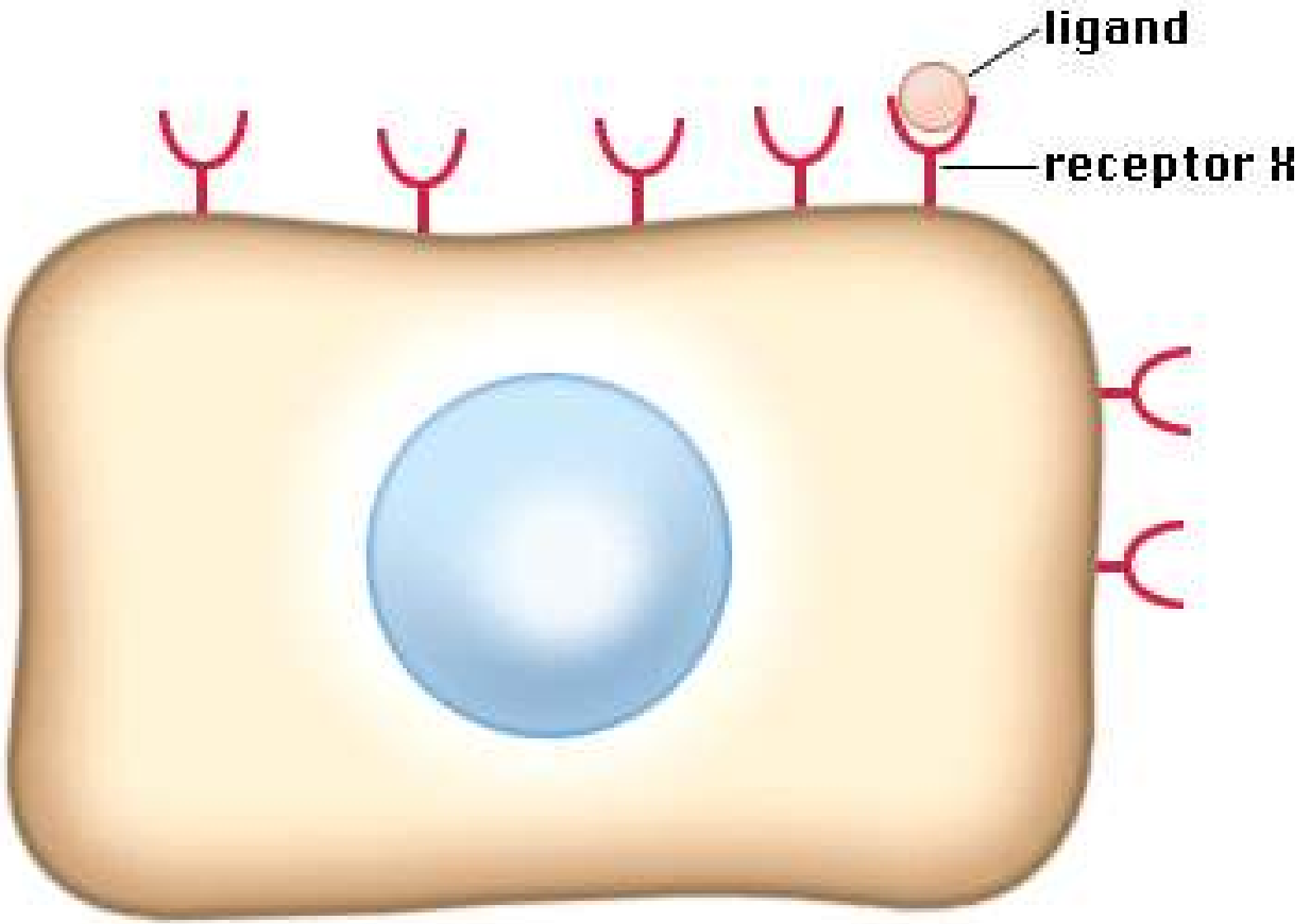


In many cases, however, the number of receptor on the cell surface is to low to use chromatography. An alternative is to produce recombinant receptor protein.





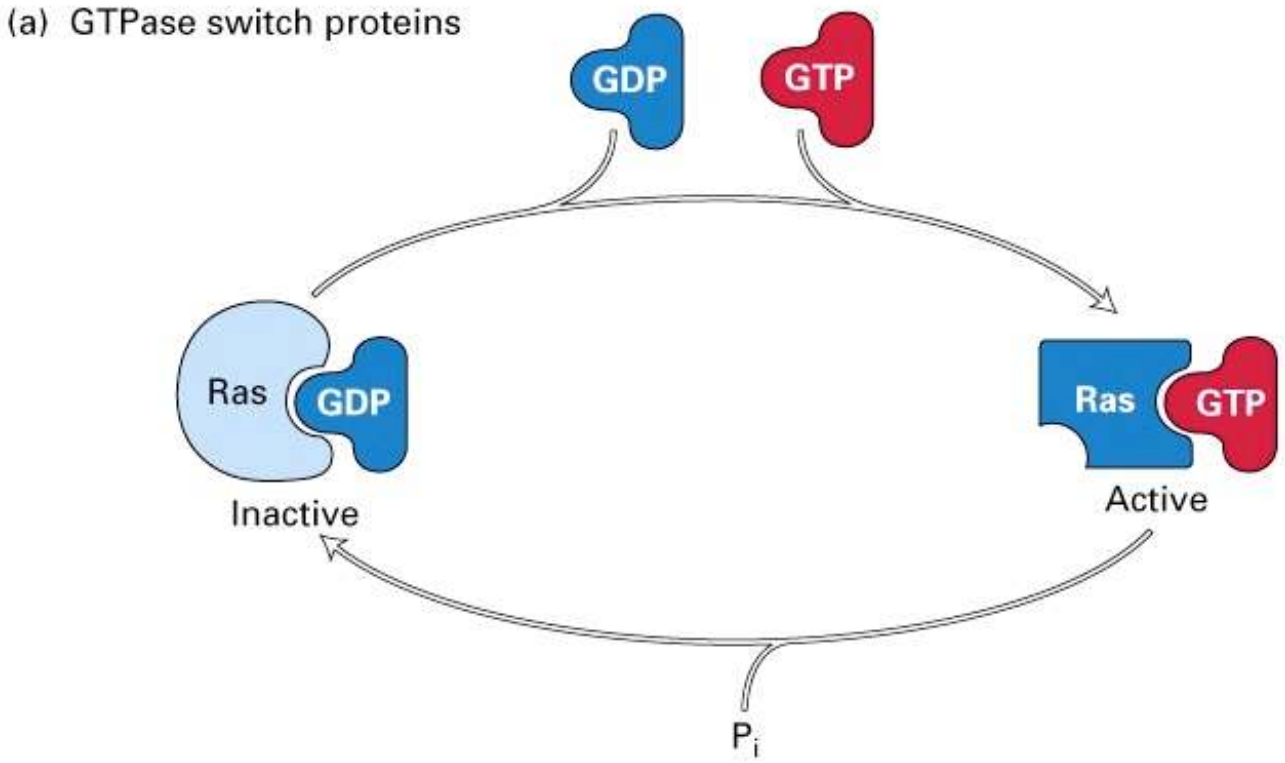
A plasmid cDNA library from the cells expressing the receptor is screen by transfecting the cloned cDNAs into cells that normally do not express the receptor. Cells that take up the cDNA encoding the receptor are detected by their binding to fluorescence or radioactively-labeled hormone. The cDNA can then be sequenced and used produce large amounts of recombinant protein.


ligand
receptor X

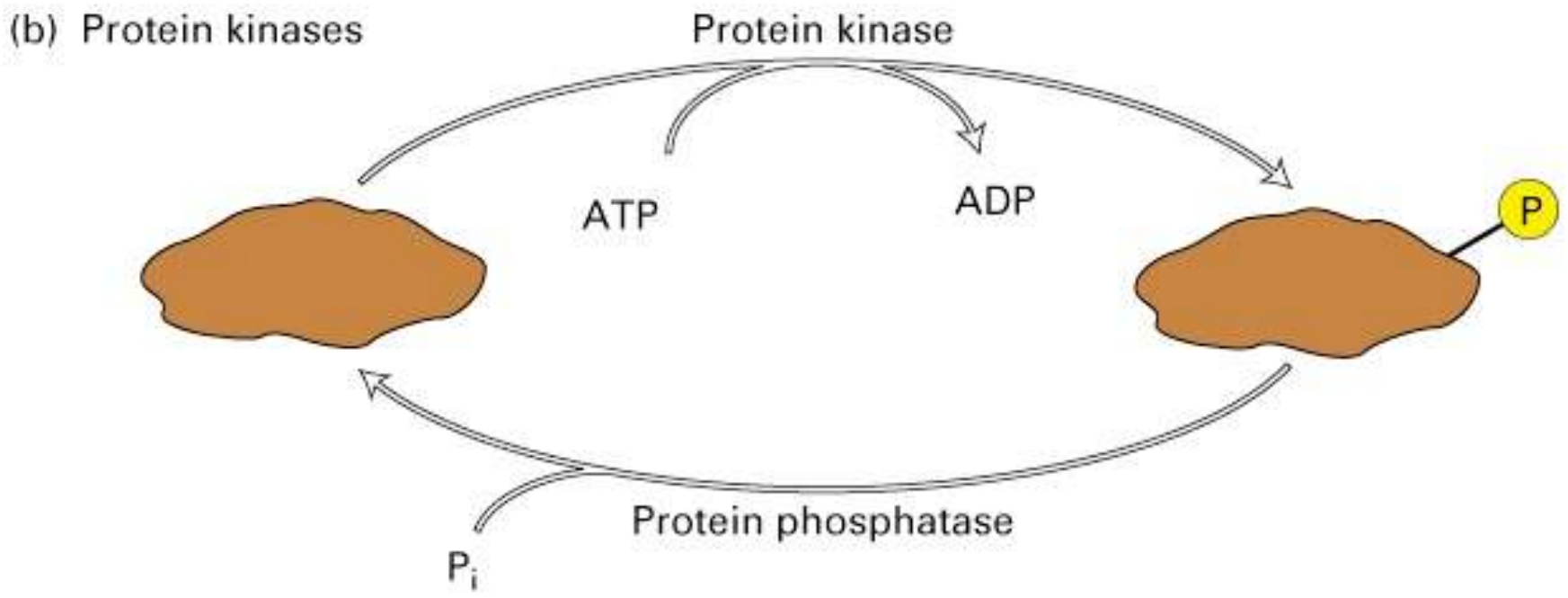
I C – Other Conserved Functions

The three main classes of intracellular signaling proteins are:

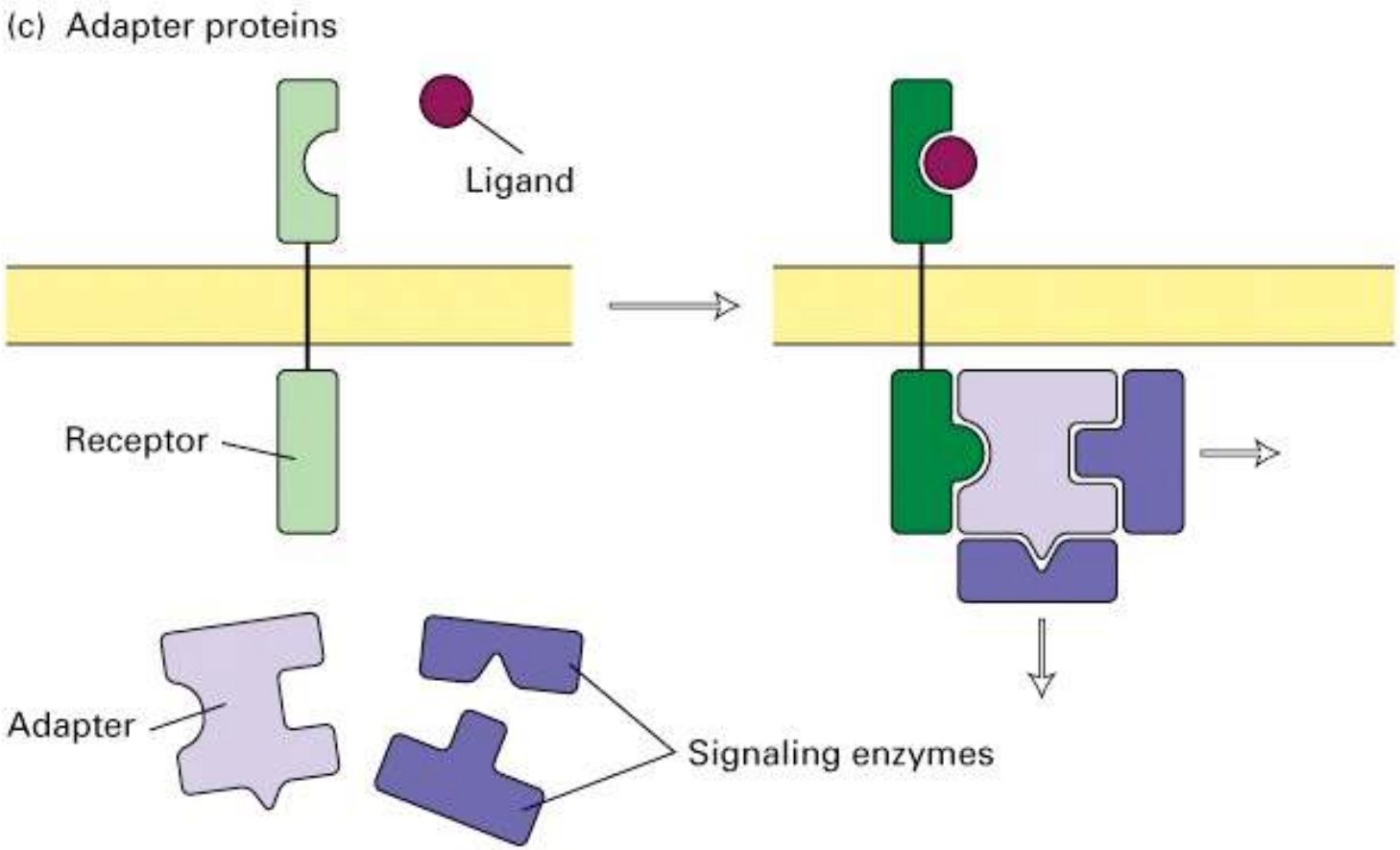
G proteins (GTPase switch proteins) - These proteins change between an active conformation when bound to GTP, and an inactive conformation when bound to GDP. IN the absence of a signal they are bound to GDP. Signal results in the release of GDP and the binding of abundant GTP. After a short period of time they hydrolyse GTP and come back to their "off" state.



Protein Kinases - Upon activation they add phosphate groups to themselves and/or other proteins at either serine/threonine, or at tyrosine residues. Their activity can be regulated by second messengers, interaction with other proteins, or by phosphorylation itself. They are opposed by phosphatases that remove phosphate groups from specific phosphorylated proteins.

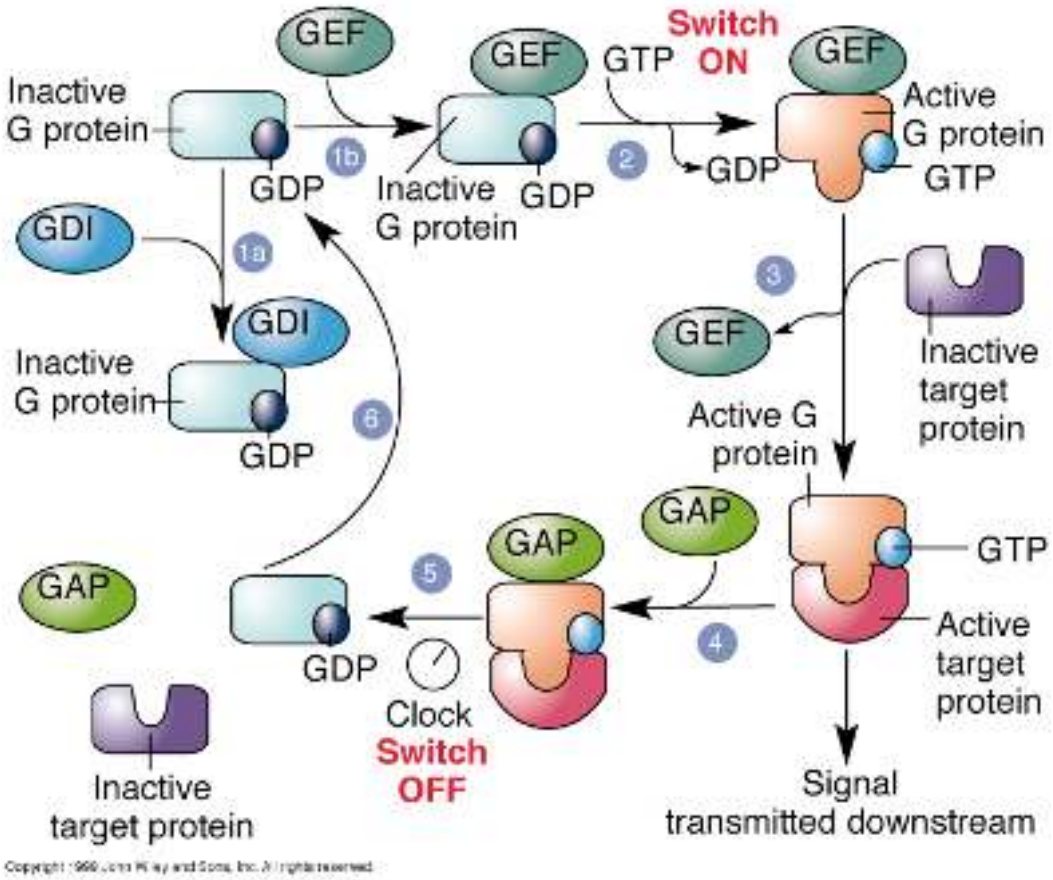


Adaptor Proteins – Many signaling pathways require the formation of large protein complexes that are held together by adaptor proteins. These proteins contain several specialized domains that act as docking sites for other proteins (e.g. SH2 domains bind to phosphotyrosines; SH3 domains bind to proline-rich sequences).



II G Proteins and G Protein-Coupled Receptors

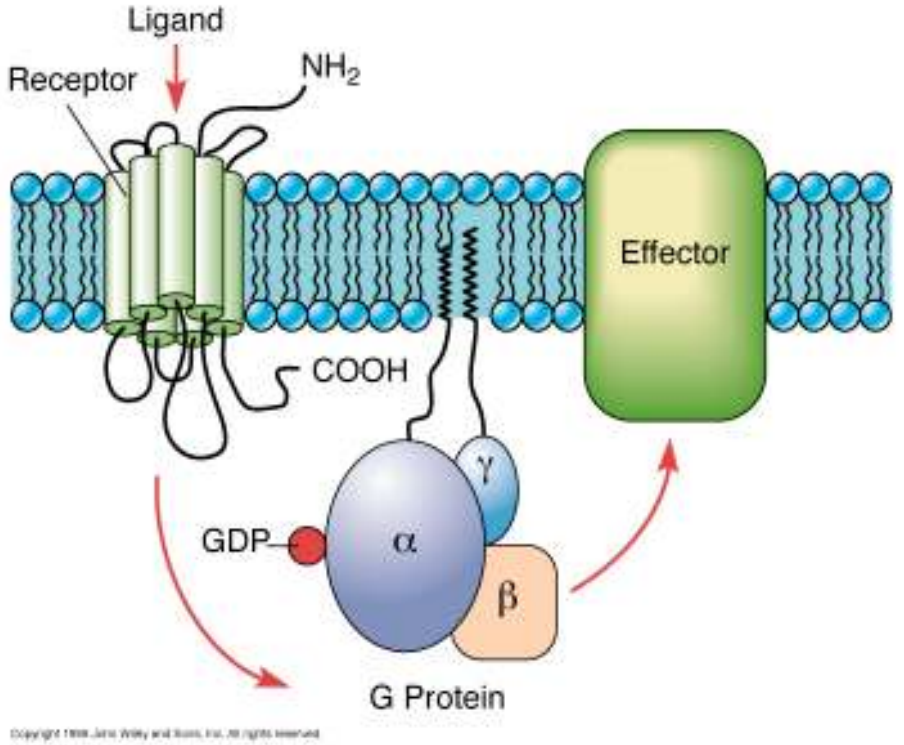
IIA - Heterotrimeric G proteins



GTP-binding proteins (or **G-proteins**) are molecular switches that turn activities on and off. In their GTP-bound state they are active and bind to specific proteins downstream, while in their GDP state they are inactive.

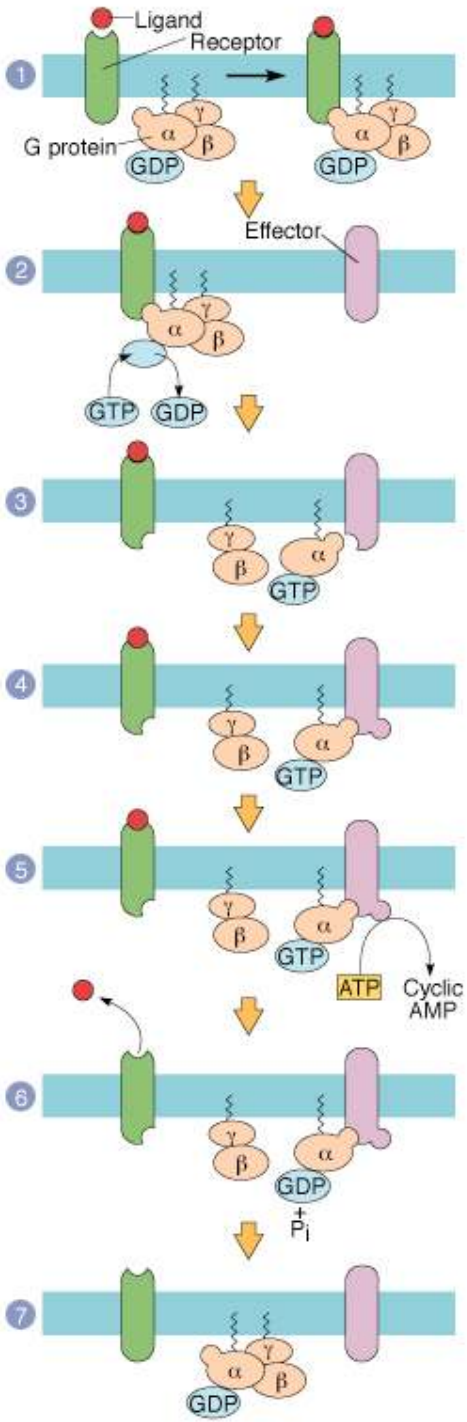
Associated proteins include GTPases-activating proteins (GAPs), guanine nucleotide-exchange factors (GEFs) and guanine nucleotide-dissociation inhibitors (GDIs)

II B - G protein-coupled Receptors



- Many different mammalian cell-surface receptors are coupled to a heterotrimeric signal-transducing G protein, covalently linked to a lipid in the membrane.
- Ligand binding activates the receptor, which activates the G protein, which activates an effector enzyme to generate an intracellular second messenger.
- All G protein-coupled receptors (GPCRs) contain 7 membrane-spanning regions with their N-terminus on the exoplasmic face and C-terminus on the cytosolic face. They are ligand specific and differ in their extracellular surface.

- GPCRs are involved in a range of signaling pathways, including light detection, odorant detection, and detection of certain hormones and neurotransmitters.
- Seven-helix transmembrane receptors and heterotrimeric G proteins exist in different isoforms: epinephrine binding to β-adrenergic receptors in cardiac muscle activates a Gs that stimulates cAMP production, inducing muscle contraction. Its binding to the α-adrenergic receptor in intestine activates a Gi that inhibits cAMP inducing muscle relaxation.



Activation of the G protein by the receptor.

Ligand binding changes the conformation of the receptor so that it binds the G protein. This in turn causes the exchange of GDP for GTP in the α subunit, switching it to the activated state. While a ligand is bound it can activate many G protein molecules.

Relay of the signal to the effector.

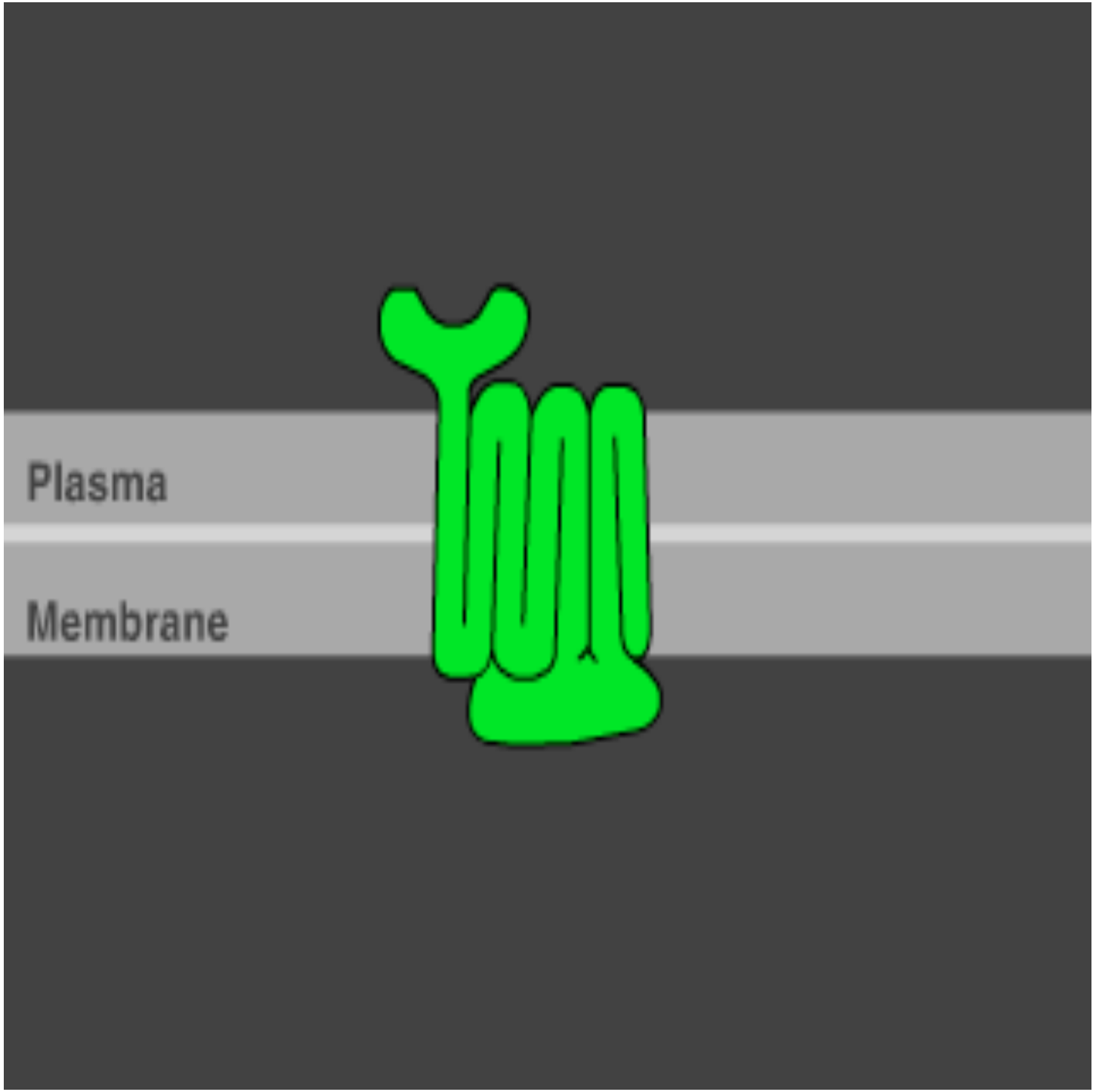
With bound GTP Gα dissociates from the G$\beta\gamma$ complex and is able to bind to its effector molecule. Binding of Gα causes the activation of the effector. G$\beta\gamma$ can in turn also activate downstream effectors.

Ending the response.

When GTP is hydrolyzed the Gα subunit rebinds to G$\beta\gamma$, returning the complex to its inactivated state. Although the Gα generally has a low GTPases activity, this can be accelerated by interaction with its GAP protein.

Deactivation (see movie)

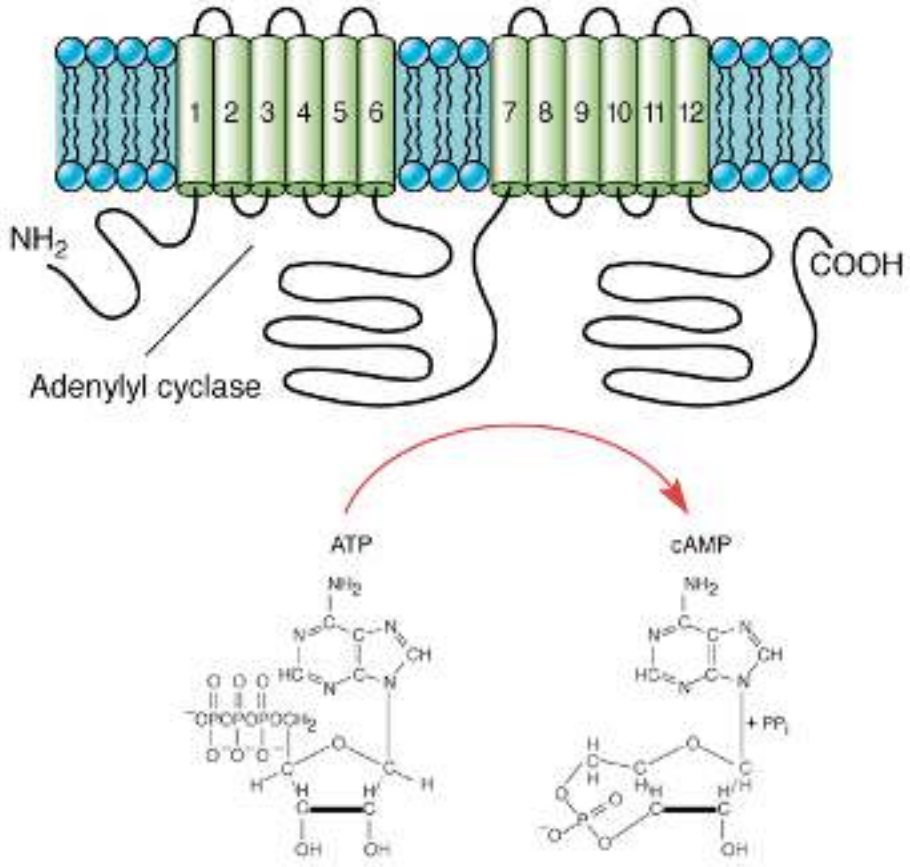
By phosphorylation and binding of arrestin


Plasma
Membrane

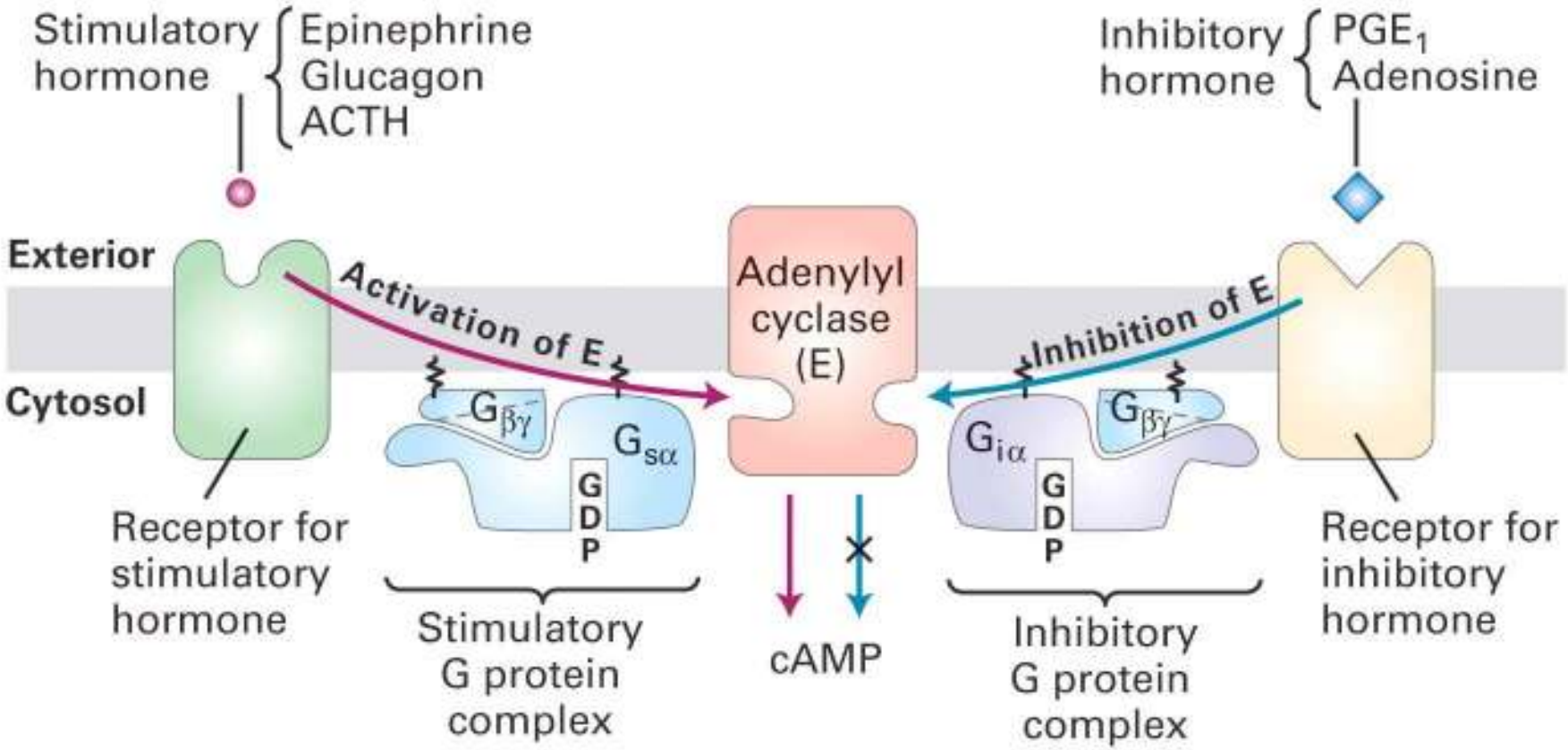
II C – Second Messenger cAMP

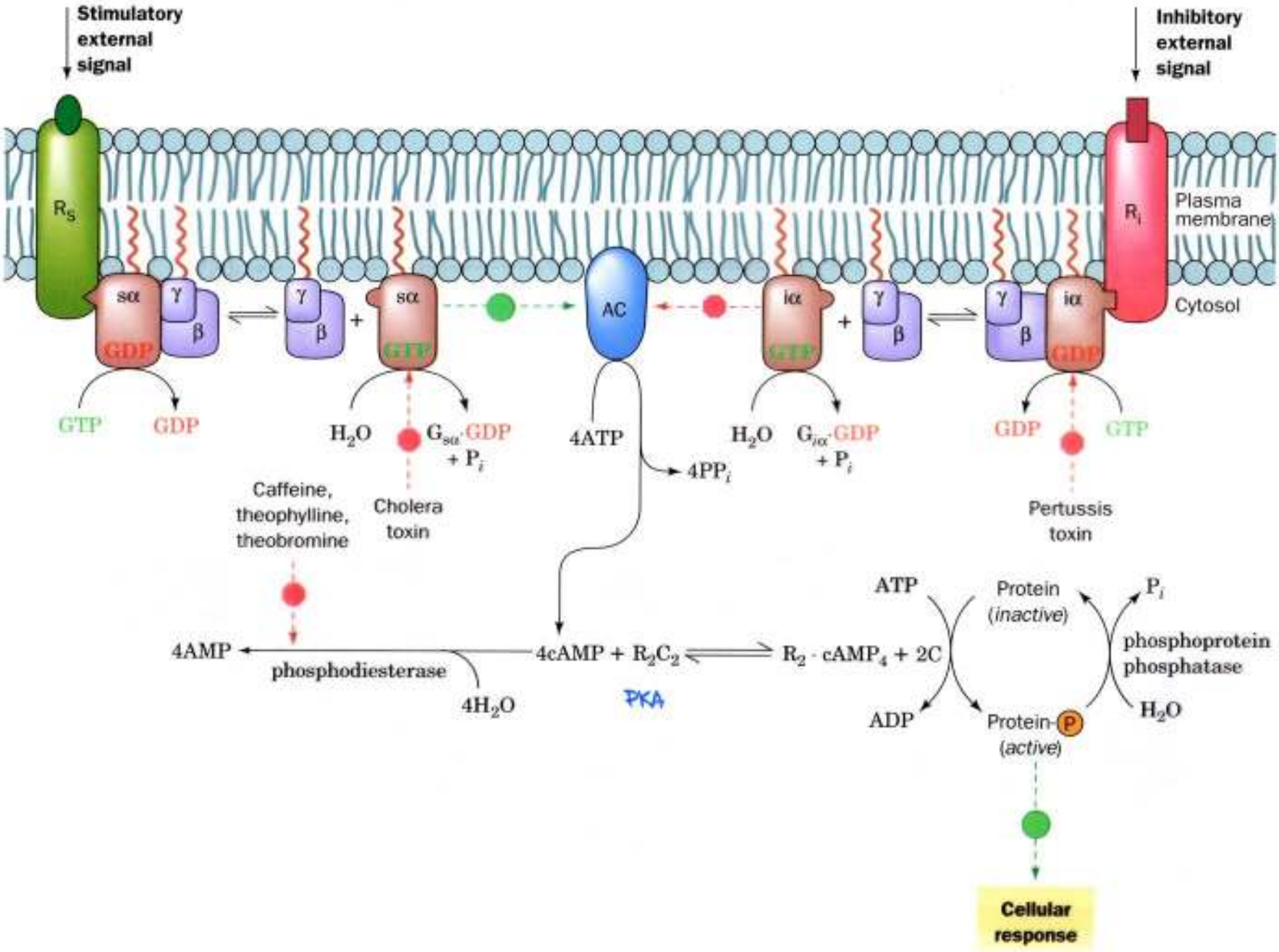
A second messenger is a substance that is released in the cytoplasm following activation of a receptor. It is non-specific and can generate a variety of responses in the cell. cAMP is an example.

cAMP is synthesized by an integral membrane protein, adenylyl cyclase, using ATP as a substrate.



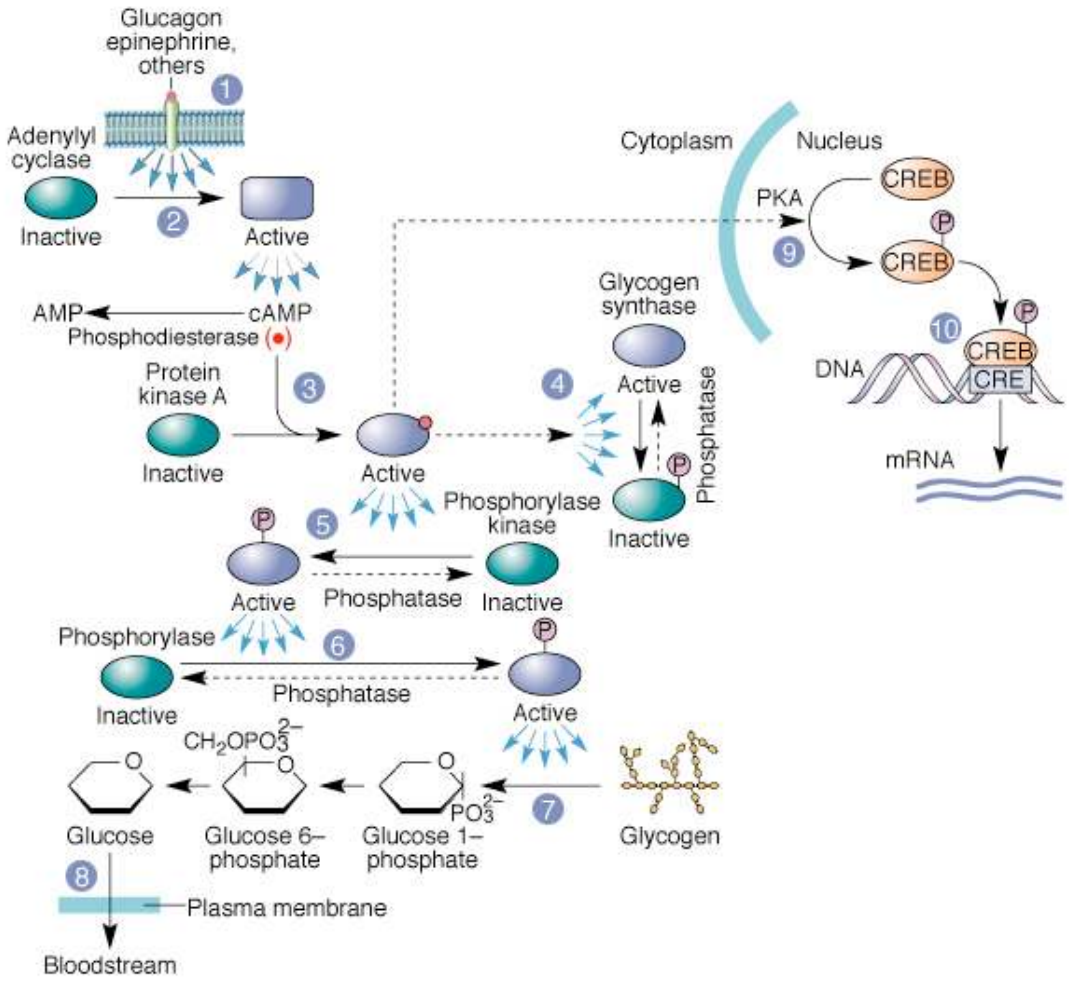
Adenylyl cyclase can be activated or inactivated by different G proteins following the binding of an inhibitory or stimulatory ligand to G protein coupled receptors.




Stimulatory external signal
Inhibitory external signal
R_S
R_i
Plasma membrane
Cytosol
sα
γ
β
GDP
GTP
AC
iα
H_2O
$G_{sα}$·GDP + P_i
$G_{iα}$·GDP + P_i
4ATP
$4PP_i$
Caffeine, theophylline, theobromine
Cholera toxin
Pertussis toxin
4AMP
phosphodiesterase
$4H_2O$
4cAMP + R_2C_2 ⇌ R_2 · $cAMP_4$ + 2C
PKA
ATP
ADP
Protein (inactive)
Protein-P (active)
P_i
phosphoprotein phosphatase
H_2O
Cellular response

Glucose Mobilization

- Glucose is stored in animal cells as glycogen, an insoluble polymer.
- The enzyme phosphorylase catalyzes glycogen breakdown, while glycogen synthase catalyzes polymerization.
- Regulation is achieved by several hormones, such as glucagon (from the pancreas) and epinephrine (from the adrenal medulla).



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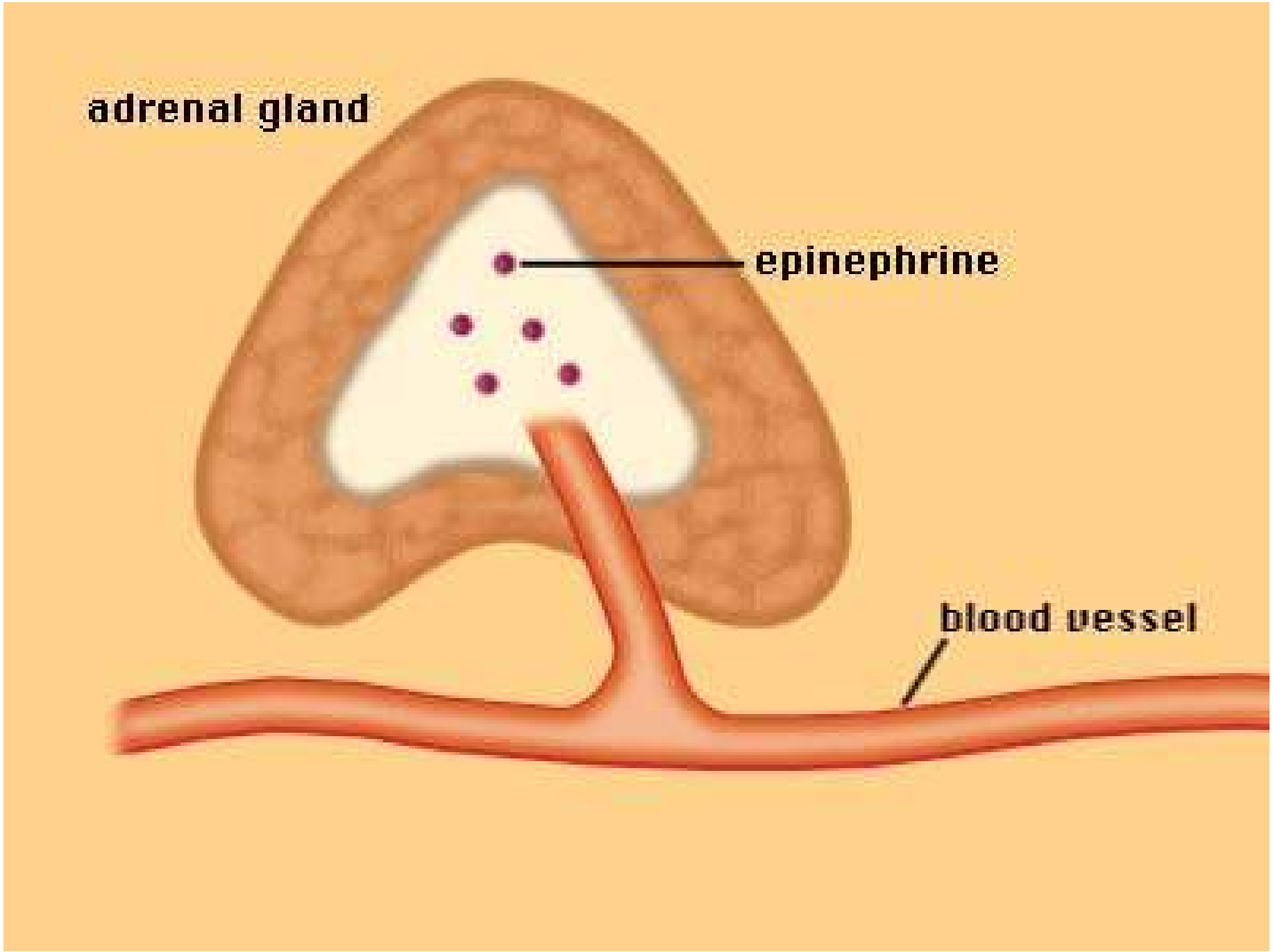
- Binding of epinephrine or glucagon to their receptors activates adenyl cyclase.
- cAMP binds to the regulatory subunit of protein kinase A (PKA) causing the release of the catalytic subunit.
- PKA phosphorylates and inhibits glycogen synthase.
- PKA phosphorylates and activates phosphorylase kinase, which then phosphorylates and activates phosphorylase.
- PKA also translocates to the nucleus where it phosphorylates the transcription factor CREB. Phosphoylated CREB binds to the CRE enhancer activating genes involved in gluconeogenesis.

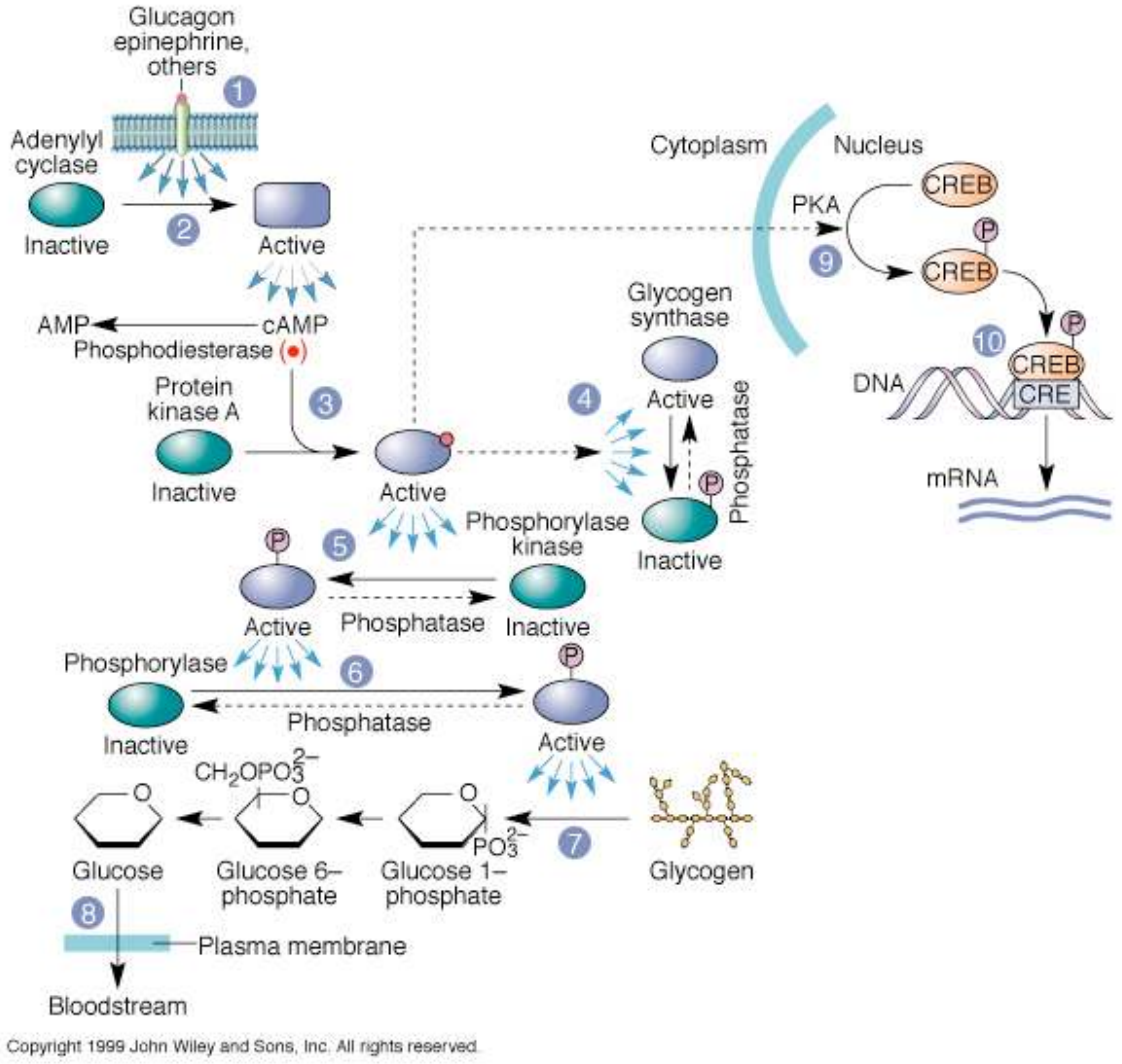

adrenal gland
epinephrine
blood vessel

Amplification

Because the concentration of hormones in the blood is very low (<10^{-8} M), the signal has to be amplified:

- binding of a single ligand stimulates a large number of adenylyl cyclases,
- each producinga large number of cAMPs. • each two cAMPs activates a PKA,
- which in term phosphorylates a number

of other proteins, and so on.



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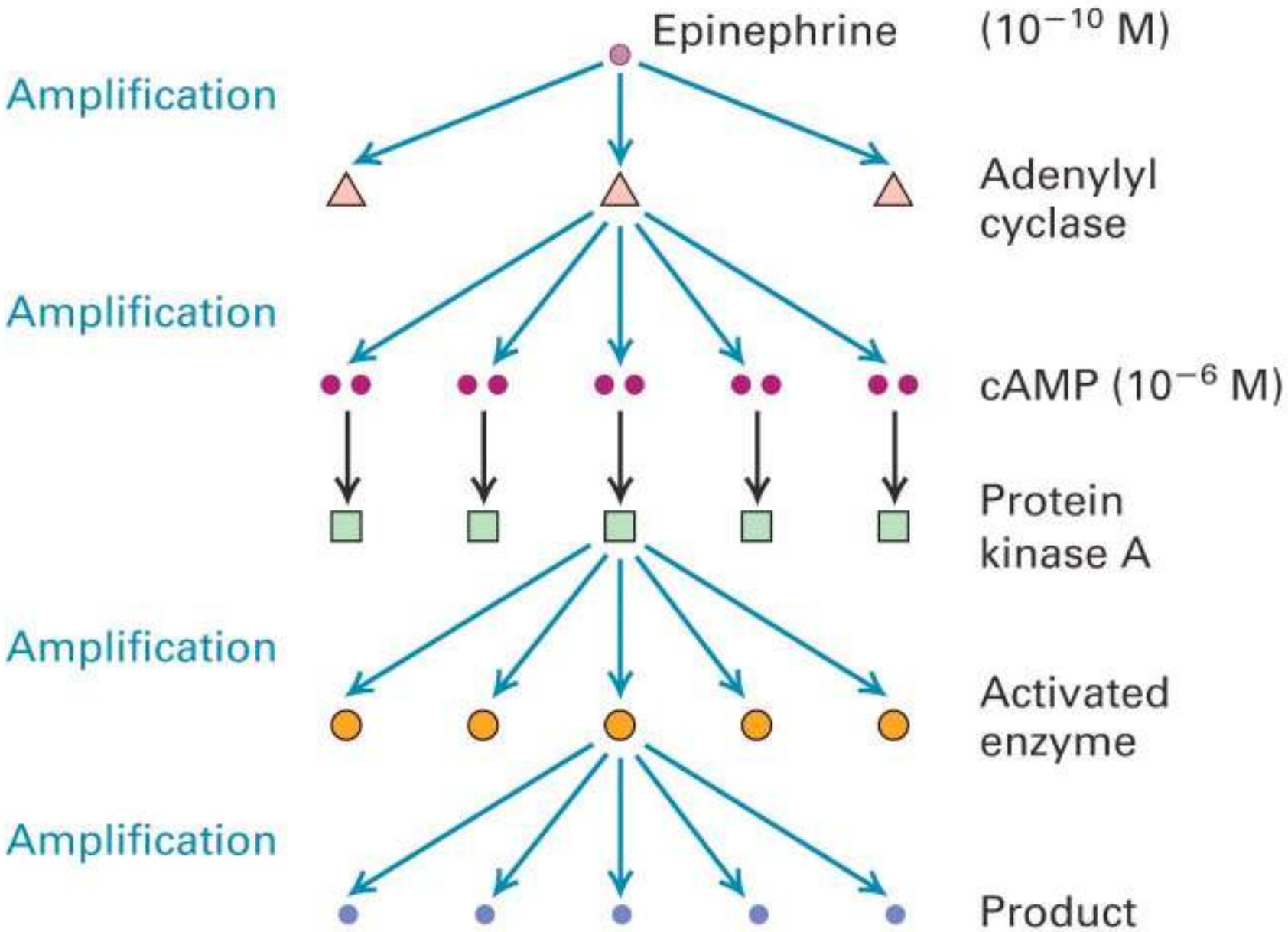
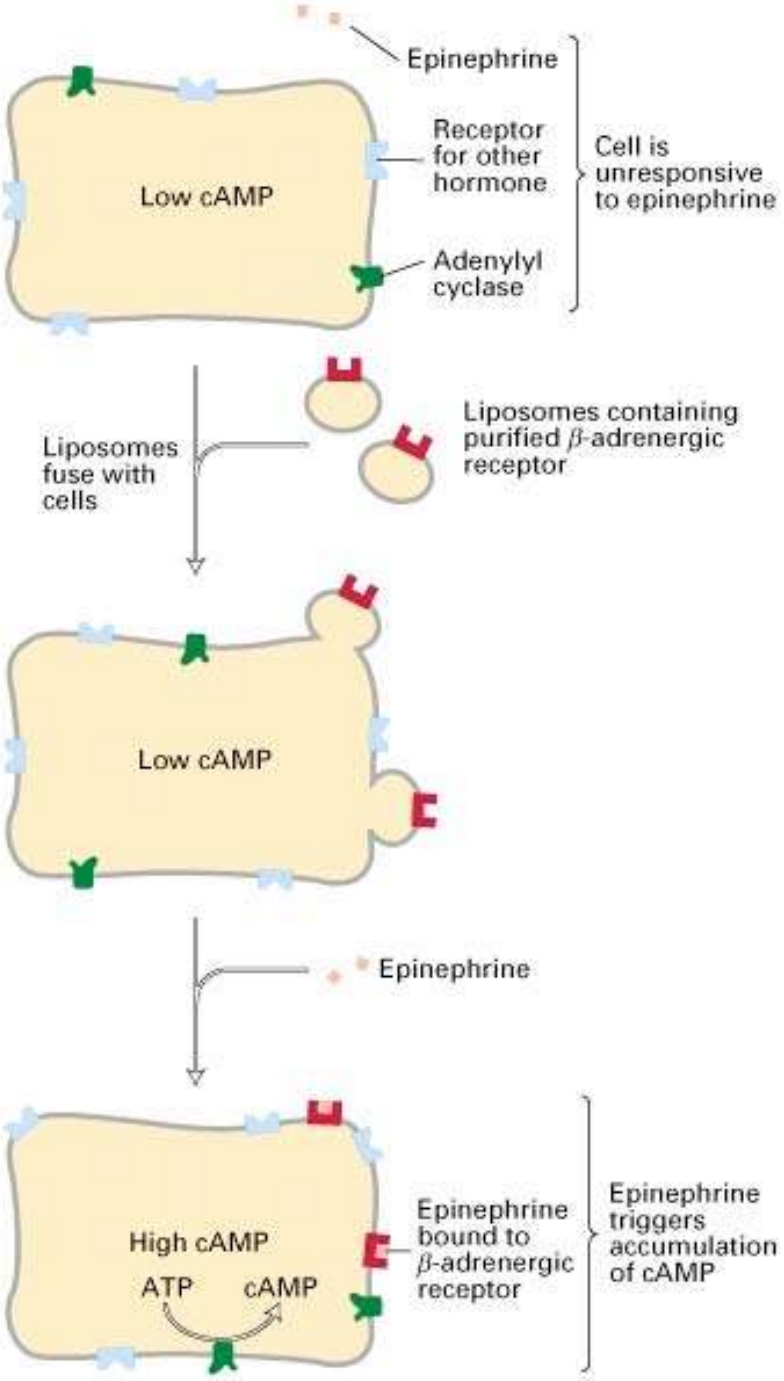
Termination

Termination of the signal involves phosphatase-1, which removes phosphates groups from the different enzymes. Its activity is regulated by inhibitor-1, which itself is activated by PKA. Because cAMP is continually degraded., when the hormone dissociates from its receptor and adenylyl cyclase is shut down, cAMP levels quickly drop. This inactivates PKA and thus inhibitor-1, leading to the activation of phosphatase-1.


Epinephrine
(10^{-10} M)
Amplification
Adenylyl cyclase
Amplification
cAMP (10^{-6} M)
Protein kinase A
Amplification
Activated enzyme
Amplification
Product



The figure on the left shows the experimental demonstration that β-adrenergic receptors mediate the induction of cAMP by epinephrine.

1 – Target cells lacking the β-adrenergic receptor but containing Adenylyl cyclase and G proteins fail to respond to epinephrine and their cytosolic cAMP remains low.

2 – Purified liposomes with the receptor are incubated with these cells and fuse, incorporating the receptor to the cell surface.

3 – When exposed to epinephrine these cells now quickly increase their cytosolic cAMP levels.

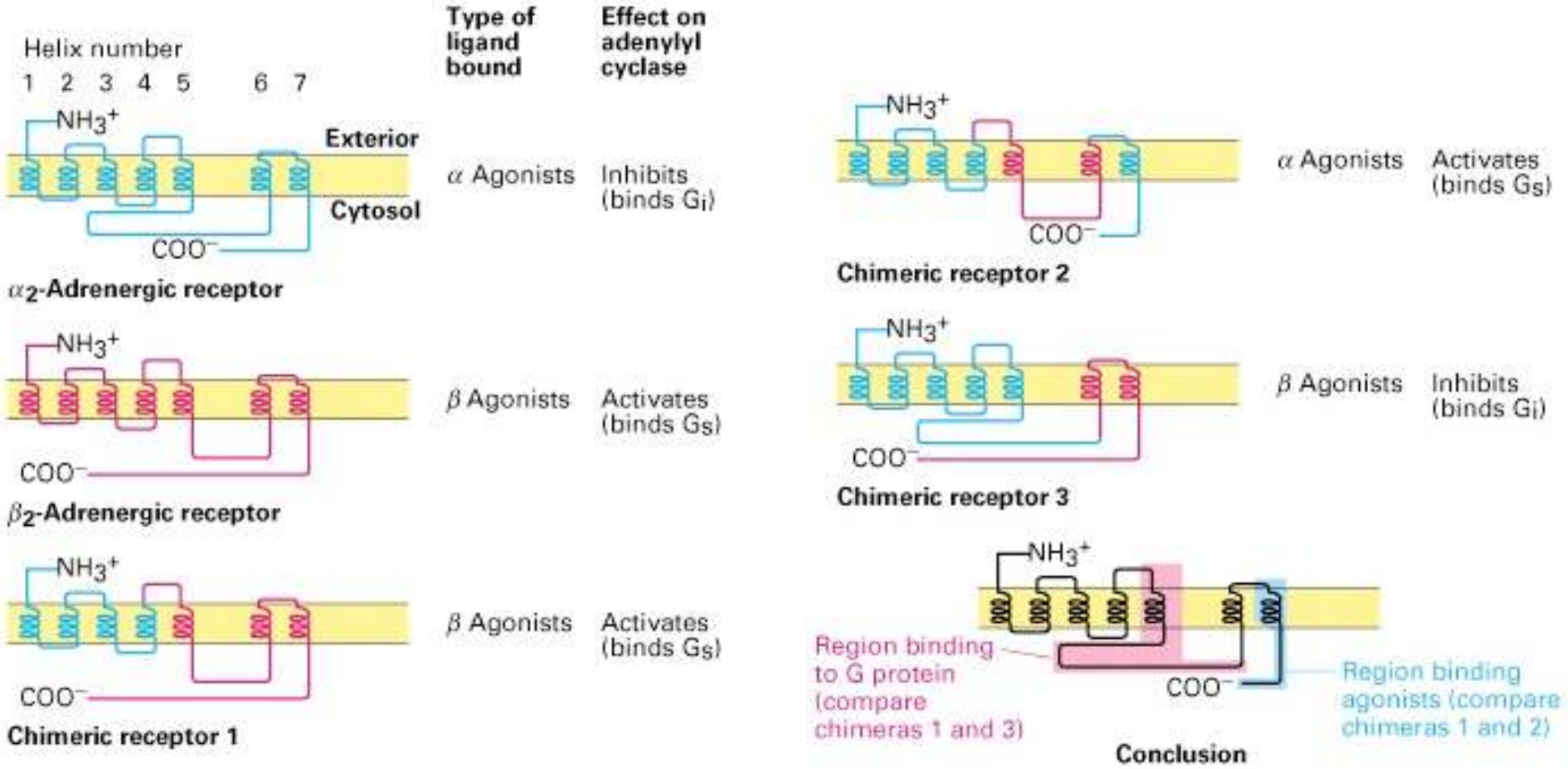
Wild type and chimeric α- and β-adrenergic receptors were expressed in Xenopus oocytes. Agonist ligands that bind selectively to one or the other where used to determined the regions of the receptor involved in ligand binding and in interaction with the G protein (Gi or Gs).

Chimera 1 acts like a β-adrenergic receptor.

Chimera 2 bindings the α agonist, but still activates cAMP (binds Gs)

Chimera 3 binds the β agonist but inhibits cAMP (binds Gi)

In conclusion, the C-terminal fragment is involved in ligand binding/recognition, while regions 5 and 6 interact with the G protein.



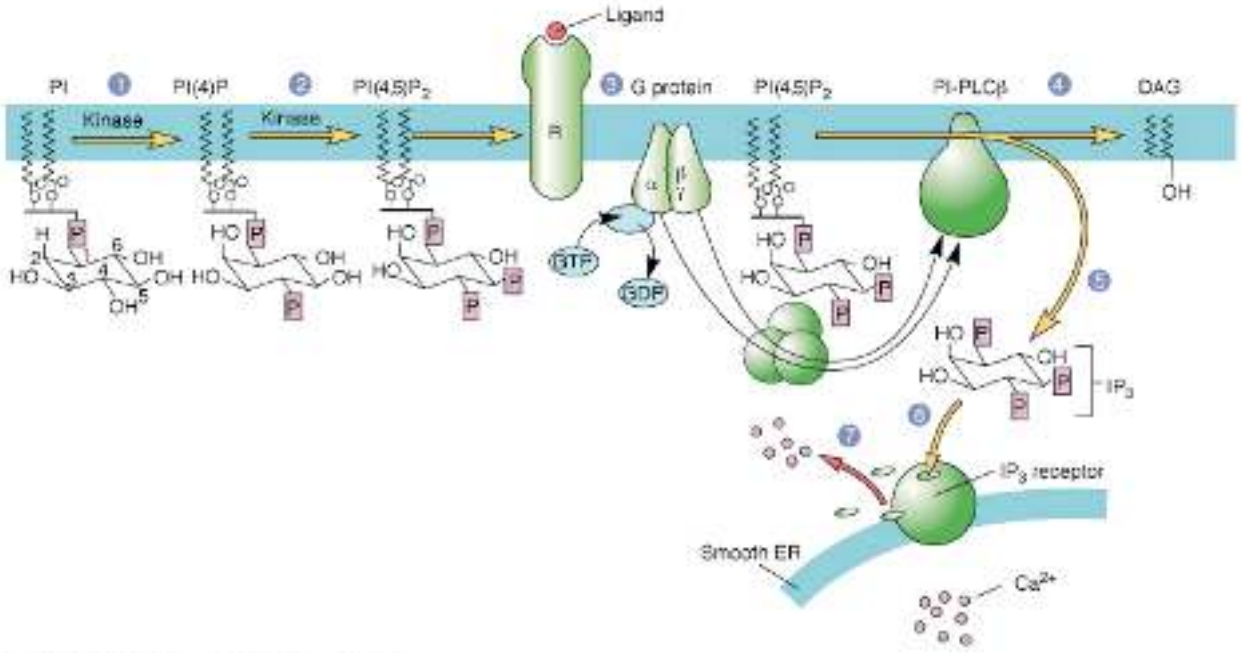
II D – Lipid-derived Second Messengers

Activated phospholipases (hydrolytic enzymes that split phospholipids) can convert certain cell membrane phospholipids into second messengers.

Phosphatidylinositol (PI)

- PI can be phosphorylated to PIP and to PIP_2 by different kinases that are themselves activated by G protein couple receptors (GPCRs) and receptor tyrosine kinases (RTKs – see later).
- PIP_2 can by cleaved by phopholipase C (PLC), which is itself activated, depending on the isoform, by GPCRs and RTKs. The two products of the cleavage, the lipophilic DAG, and the soluble IP_3, are secondary messengers in several signaling pathways.

IP_3 binds to a IP_3-gated Ca^{++} channel in the ER membrane that opens upon IP3 binding increasing cytosolic Ca^{++} concentrations.



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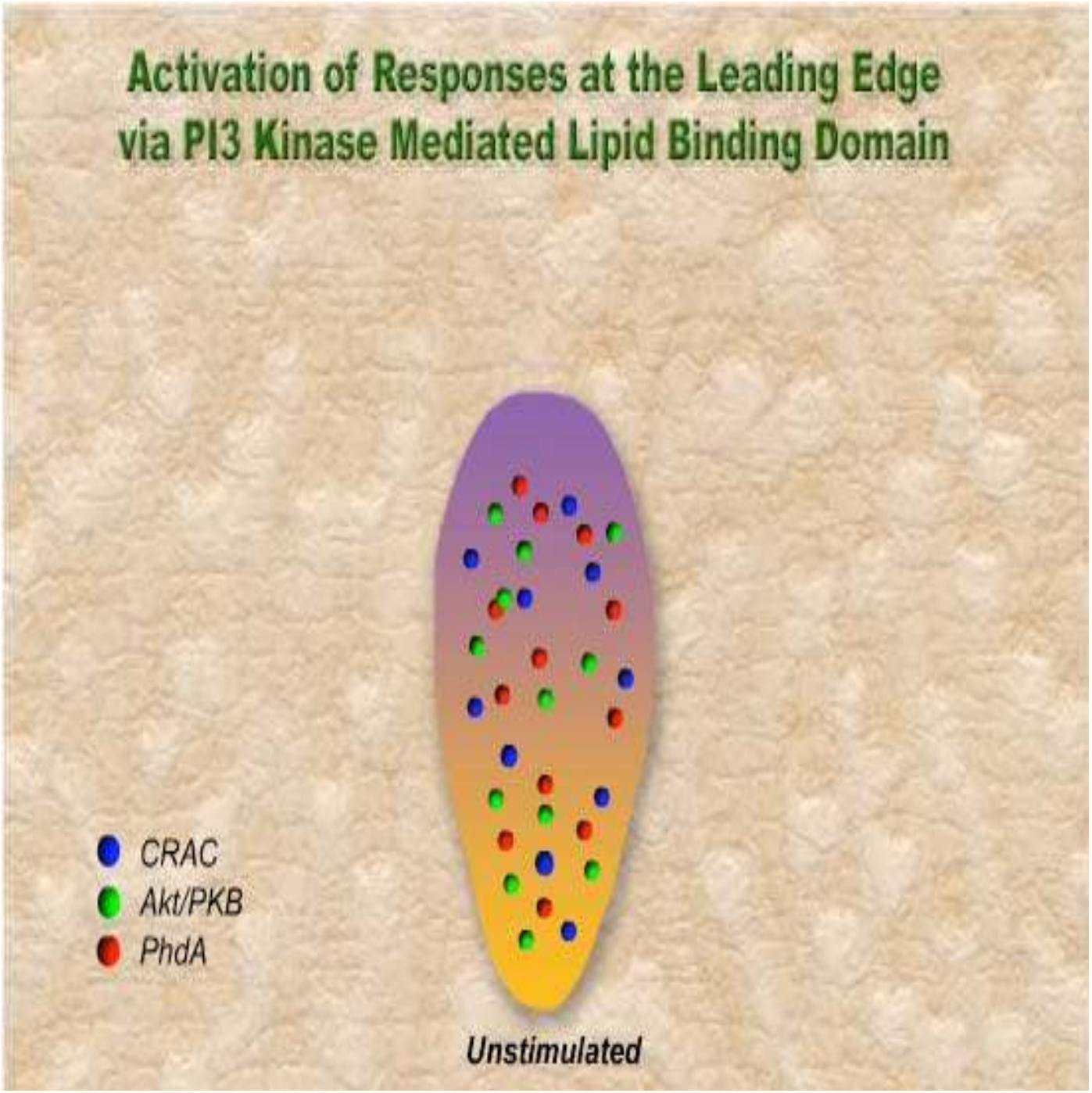
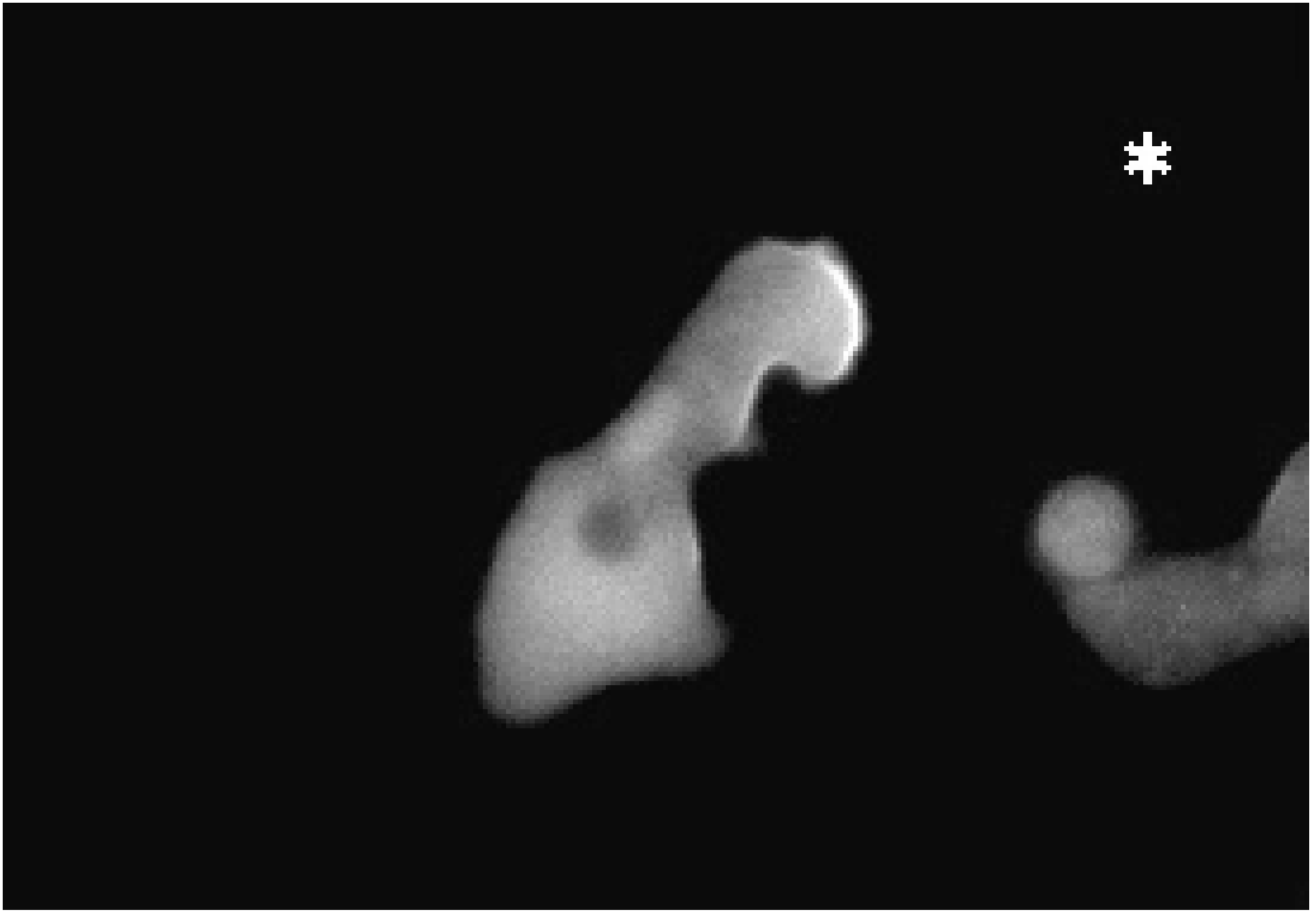
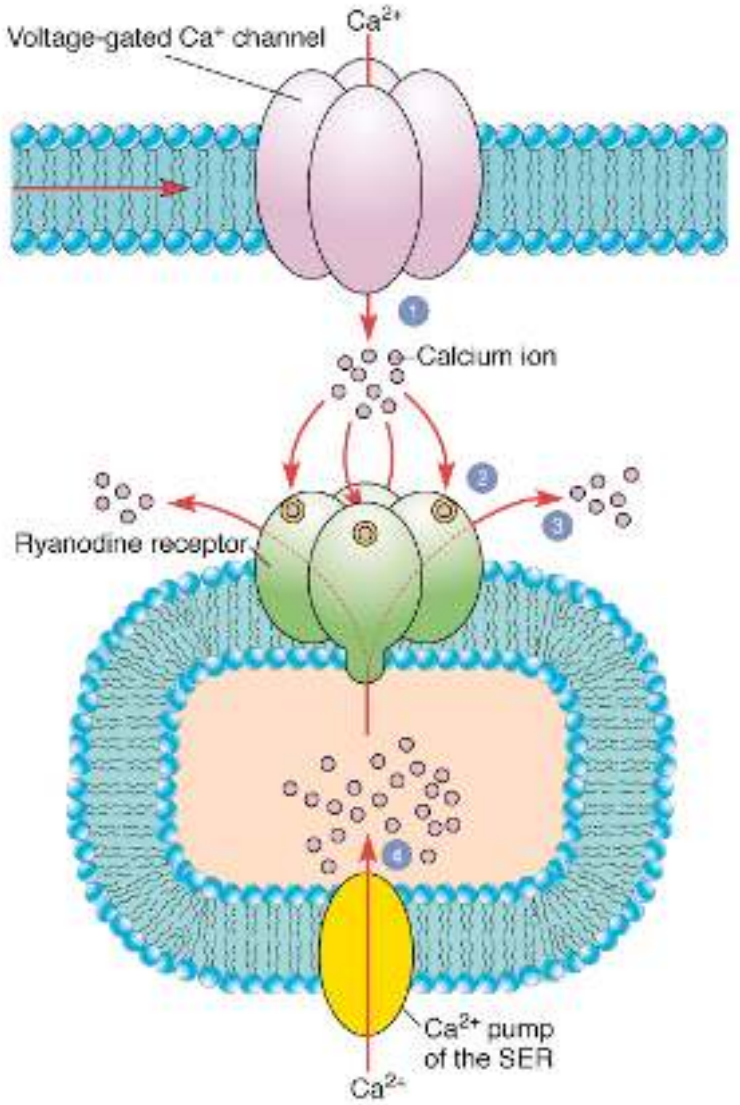

Activation of Responses at the Leading Edge
via PI3 Kinase Mediated Lipid Binding Domain
CRAC
Akt/PKB
PhdA
Unstimulated



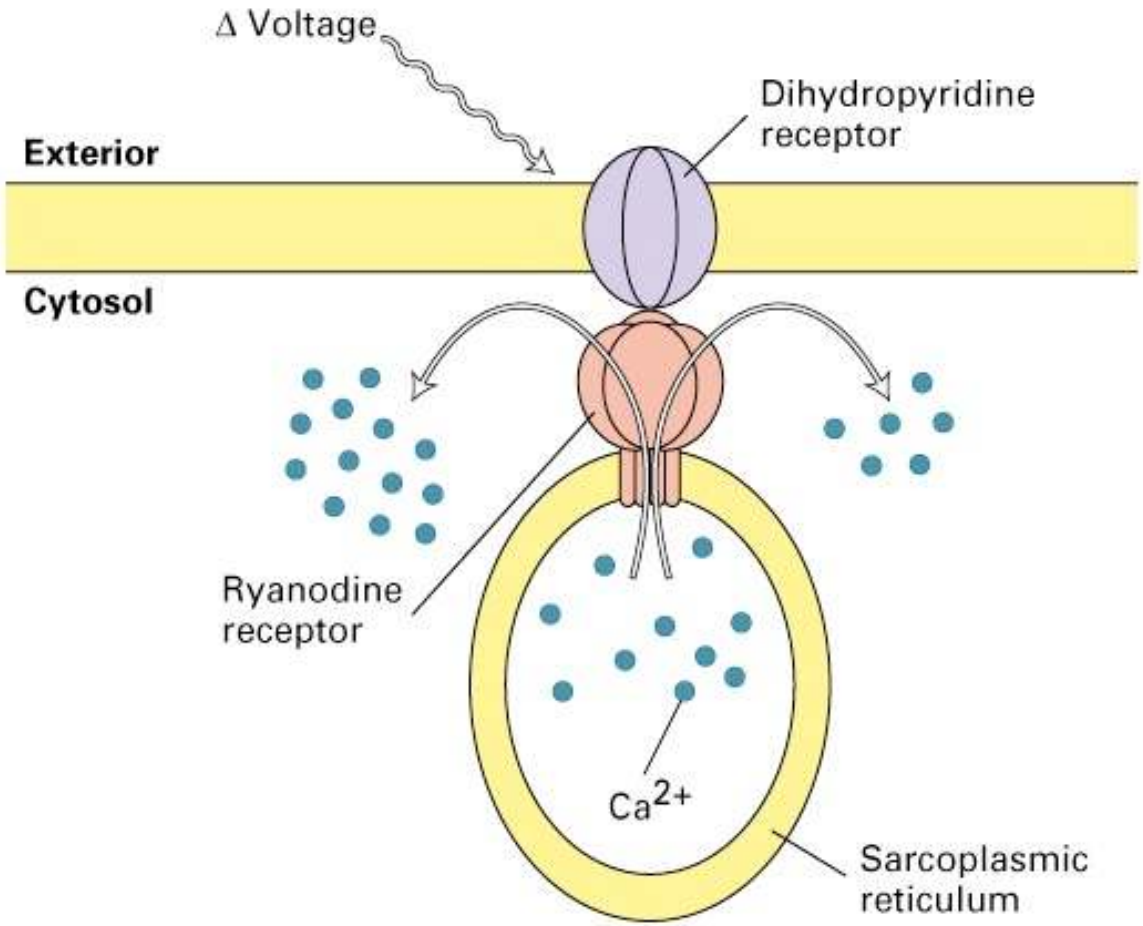
II E – Calcium Signaling

The concentration of Ca^{++} is kept very low in the cytosol (10^{-7} – 10^{-8} M), 10,000 times lower than in the extracellular space, by means of Ca^{++} pumps that pump these ions out of the cell or into the ER.

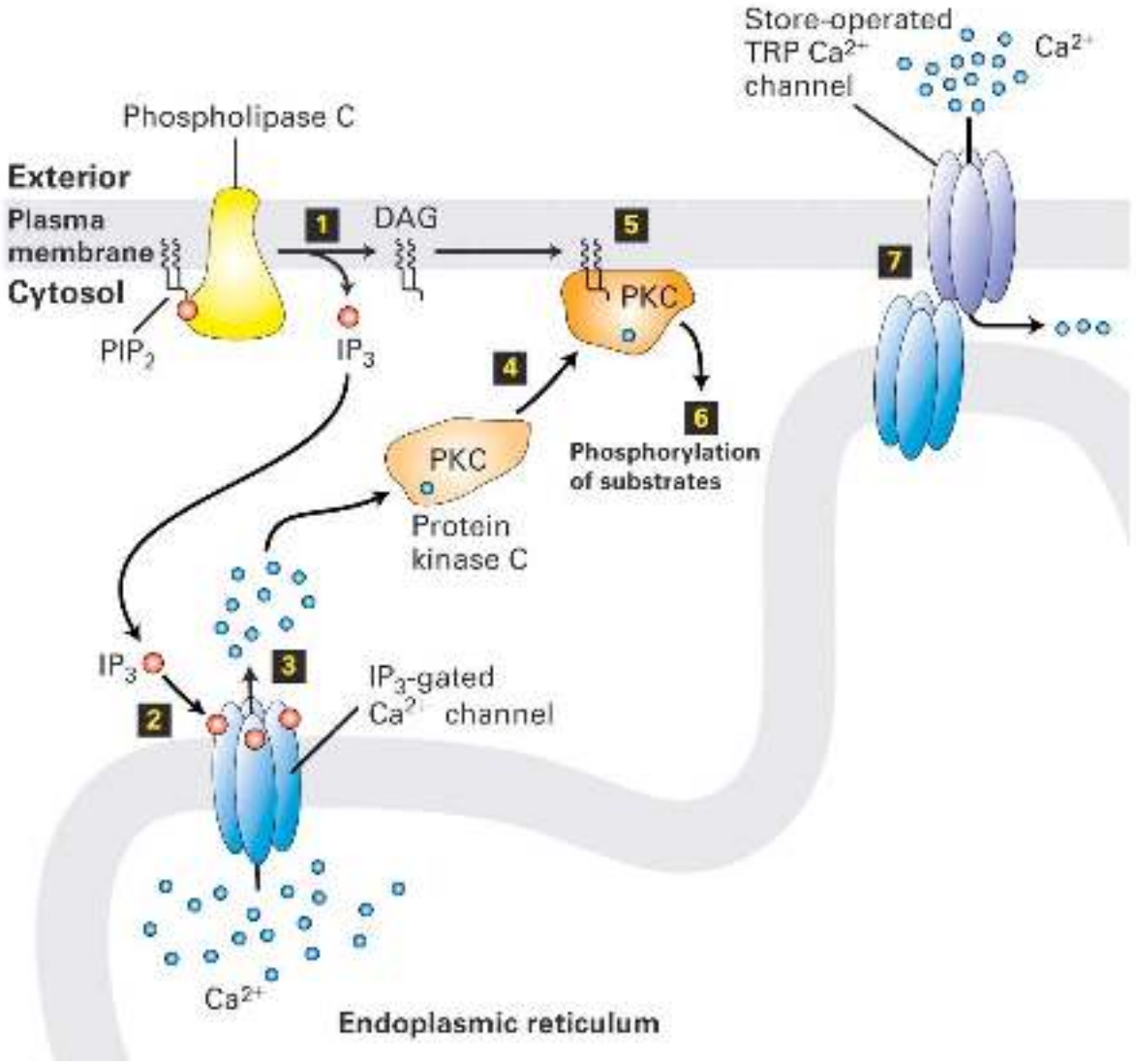
Regulated Ca^{++} channels open upon different stimulae to transiently let Ca^{++} into the cytosol where it serves as a secondary messenger in several signaling pathways.



The main types of Ca^{++} channels are the IP_3-receptor discussed above and the ryanodine receptor. The latter exists in the ER of nerve cells and the sarcoplasmic reticulum of muscle cells where, upon arrival of the action potential, opens to trigger calcium influx into the cytosol that results in muscle contraction. Ryonodine receptors are always in close proximity to voltage-gated Ca^{++} channels and other voltage-sensitive receptors in the plasma membrane that are involved in their activation.



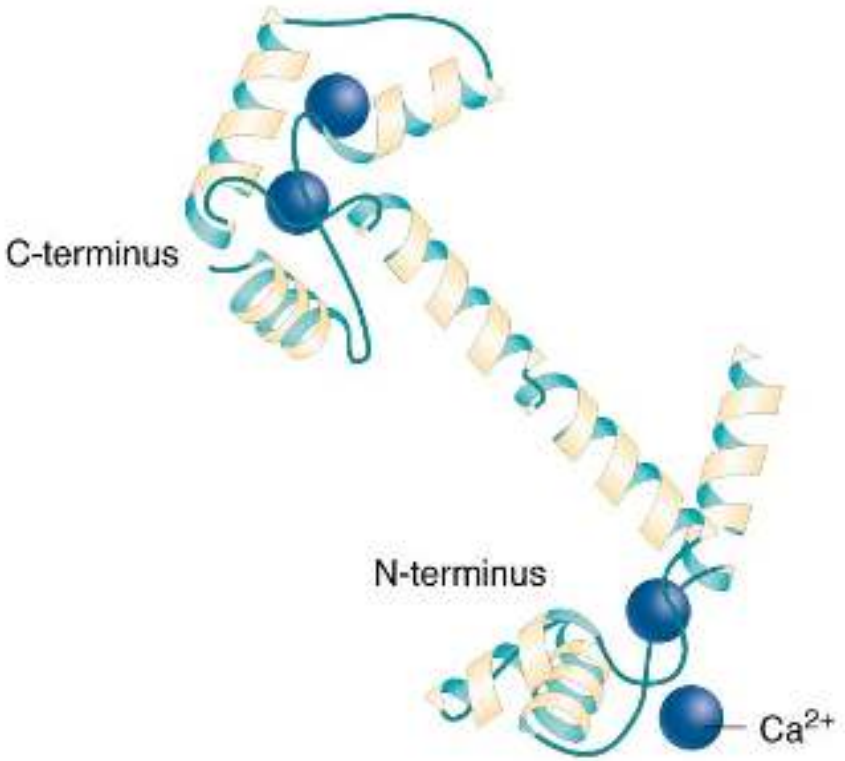
Ca^{++} is involved in a number of signaling pathways. Elevation of Ca^{++} levels via the inositol-lipid signaling pathway involves protein kinase C (PKC). Binding of Ca^{++} to the kinase recruits it to the membrane where its kinase activate is stimulated by interaction with DAG. PKC then phosphorylates a number of substrates further along the pathways.



Ca^{++} effects includes exocytosis of secretory vesicles, muscle contraction or the inducement of mitosis in fertilized eggs.

To trigger these responses calcium affects a number of cellular effectors. In most cases it does it in conjunction with the calcium-binding protein **calmodulin**. This protein is found in all eukaryotes and is widely conserved in sequence among species.

When the concentration of Ca^{++} increases calmodulin binds 4 Ca^{++} ions. This results in a large conformational change in the protein that increases its affinity for a number of effector proteins.



(a)

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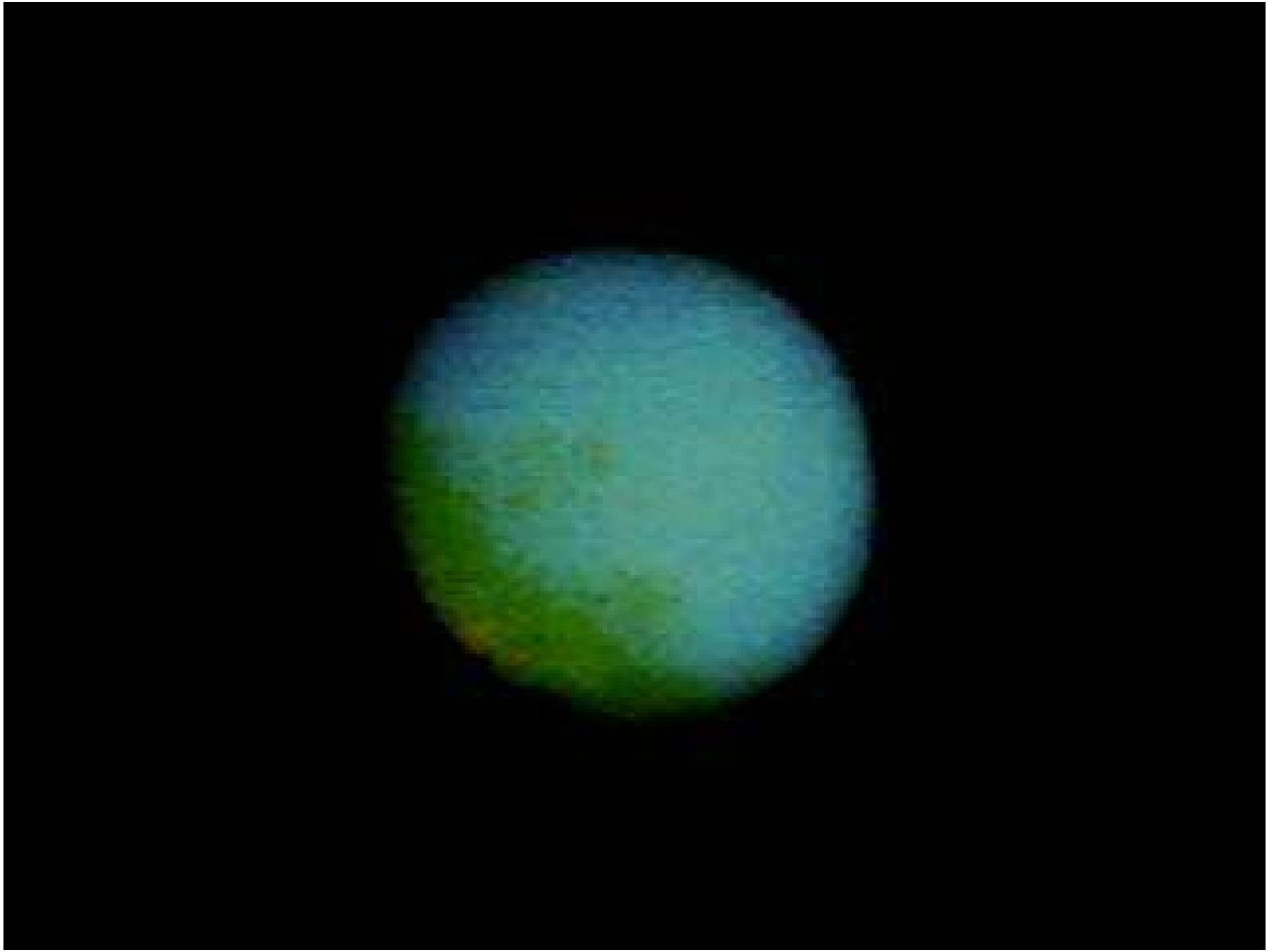
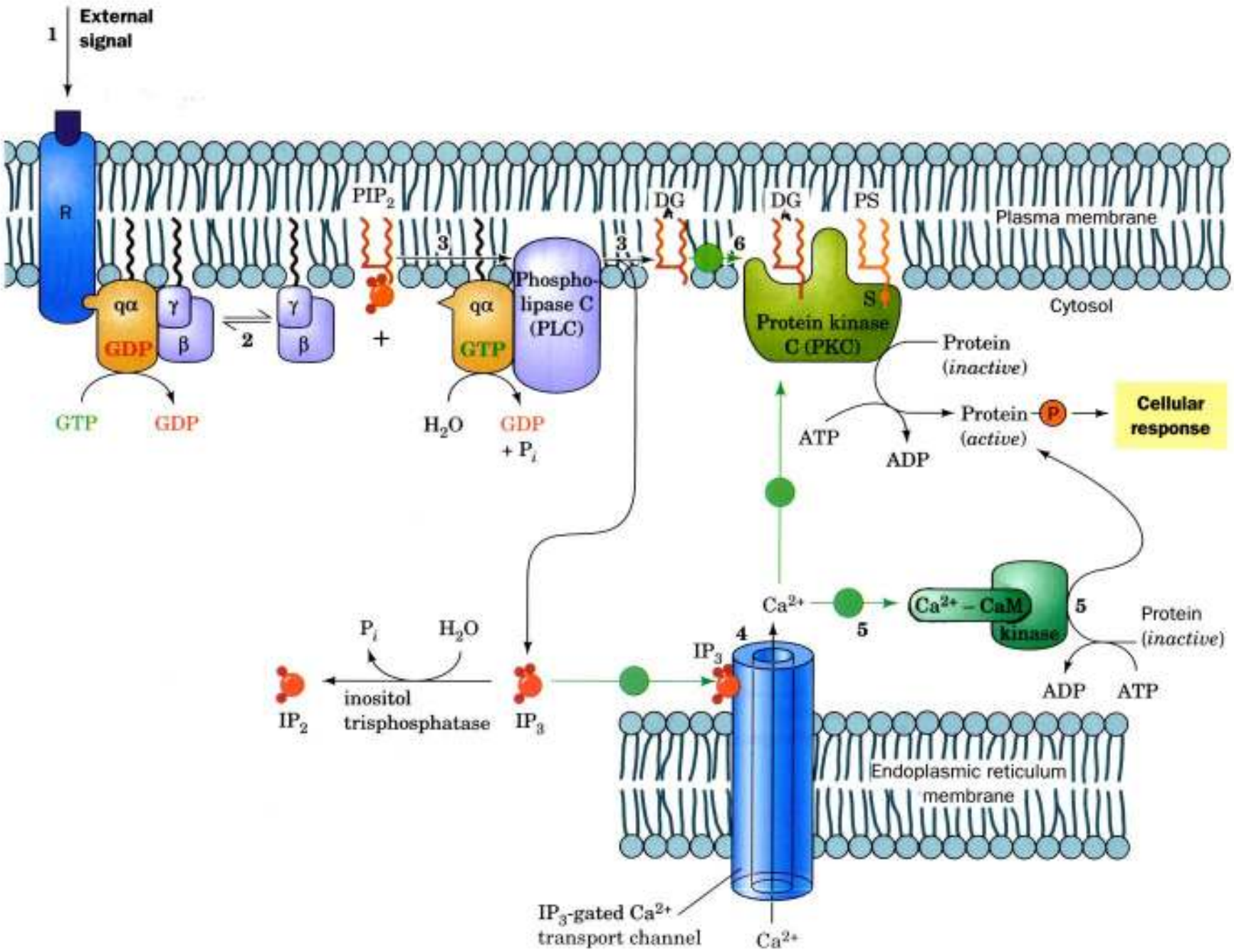
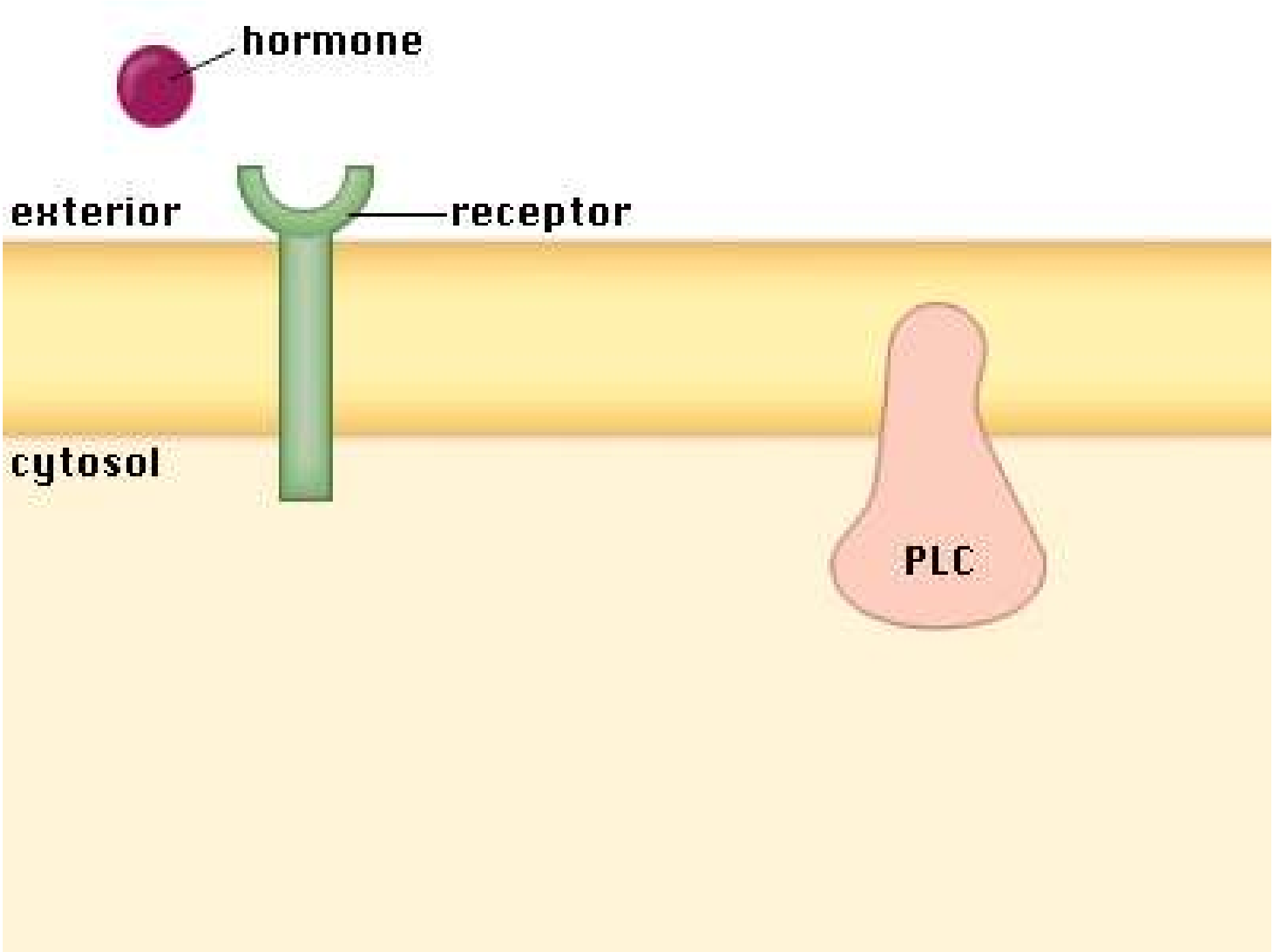




1
External signal
R
PIP2
DG
DG
PS
Plasma membrane
3
3
6
qα
GDP
γ
β
2
γ
β
+
qα
GTP
Phospho-lipase C (PLC)
S
Protein kinase C (PKC)
Cytosol
Protein (inactive)
GTP
GDP
H2O
GDP + Pi
ATP
ADP
Protein (active)
P
Cellular response
Ca2+
5
Ca2+ – CaM kinase
5
Protein (inactive)
ADP
ATP
Pi
H2O
4
IP3
inositol trisphosphatase
IP2
IP3
Endoplasmic reticulum membrane
IP3-gated Ca2+ transport channel
Ca2+


hormone
exterior
receptor
cytosol
PLC

III Receptor Tyrosine Kinases

III A - Insulin Signaling

• Insulin signals the removal of glucose from the blood and synthesis of glycogen.

• The insulin receptor is a member of the tyrosine kinase superfamily, generally involved in cell growth and differentiation.

•The insulin receptor tyrosine kinase (RTK) is a tetramer of two extracellular α subunits and two transmembrane β subunits with a single transmembrane region.

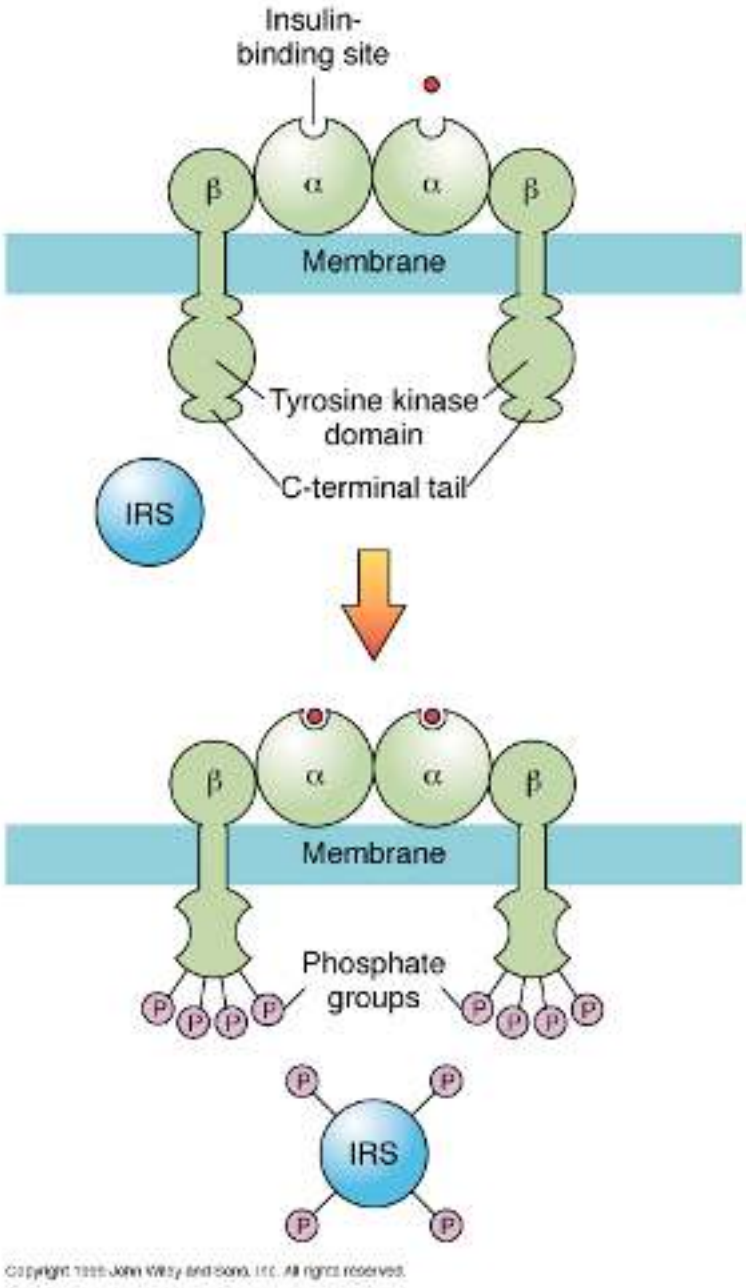
• α subunits bind insulin and induce a conformational change in the β subunits, activating their cytoplasmic kinase domain.

• Activated β subunits phosphorylate one another and a variety of insulin receptor substrates (IRSs).

• RTKs phosphorylate only phosphotyrosine motifs. After phosphorylation these motifs have a high affinity for SH2 domains.

• Interaction of phosphotyrosine motifs with SH2 proteins cause their conformational change so that they bind to other proteins or are translocated to other parts of the cell.

• Different SH2 proteins will activate separate signaling pathways.



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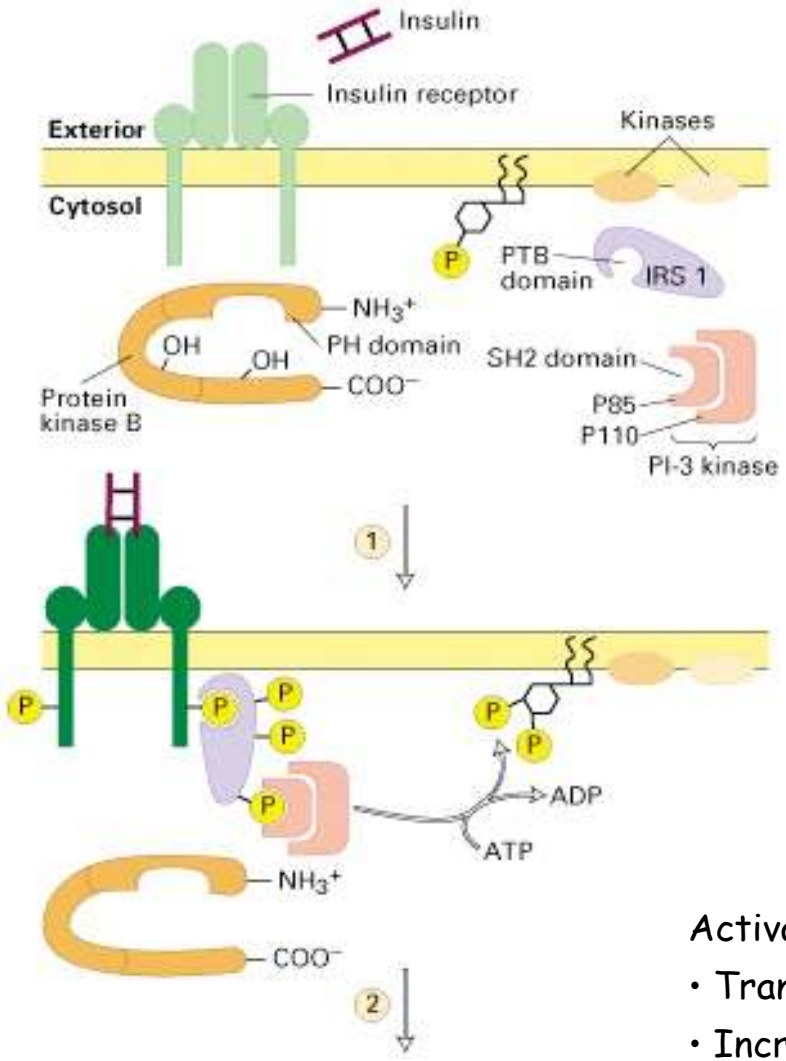
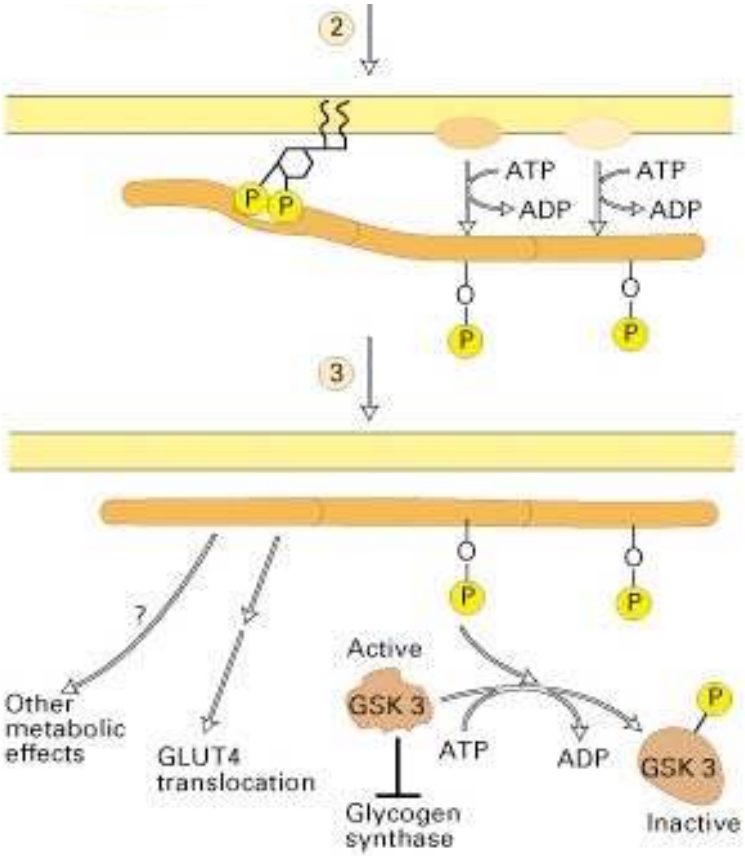
Insulin stimulation activates Protein Kinase B and MAP kinase

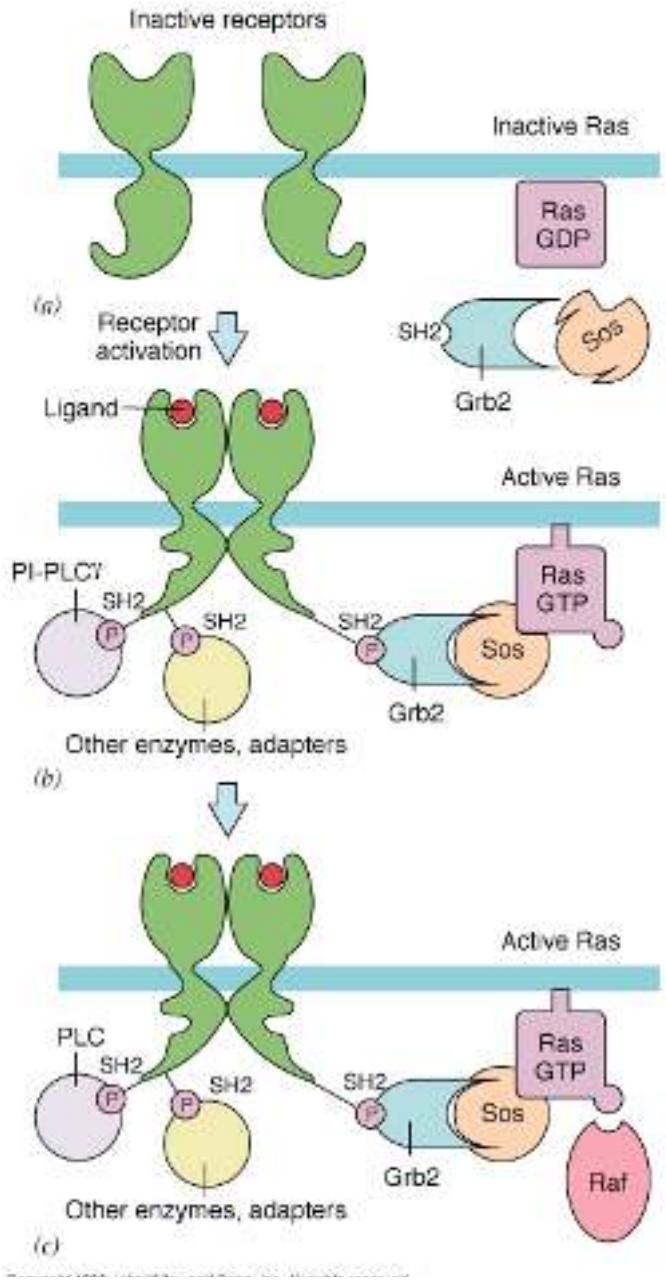




Activation of insulin RTKs results in:

- Transfer of glucose transporters to the plasma membrane
- Increase in protein synthesis
- Stimulation of Glycogen synthases
- Activation of phosphatase 1

III B - General RTKs, Ras and the MAP kinase cascade



RTKs are receptors for a variety of extracellular ligands:

- Hormones: insulin, growth hormone
- Growth factors like EGP
- Cytokines (secreted by certain immune cells to affect others: interferones, interleukins)

• Most RTKs are monomers that only dimerize when bound to their ligand.

• Dimerization activates the kinase activity and leads to autophosphorylation, creating sites for interactions with specific effectors. A key component of the RTK cascade is Ras

Ras

• Ras is small, monomeric G protein with very low GTPase activity on its own. GAPs activate hydrolysis in Ras by 10^5 fold.
• Ras is mutated in 30% of human tumors. Most mutations block hydrolysis keeping Ras active and the cell in a proliferative state.

Grb2

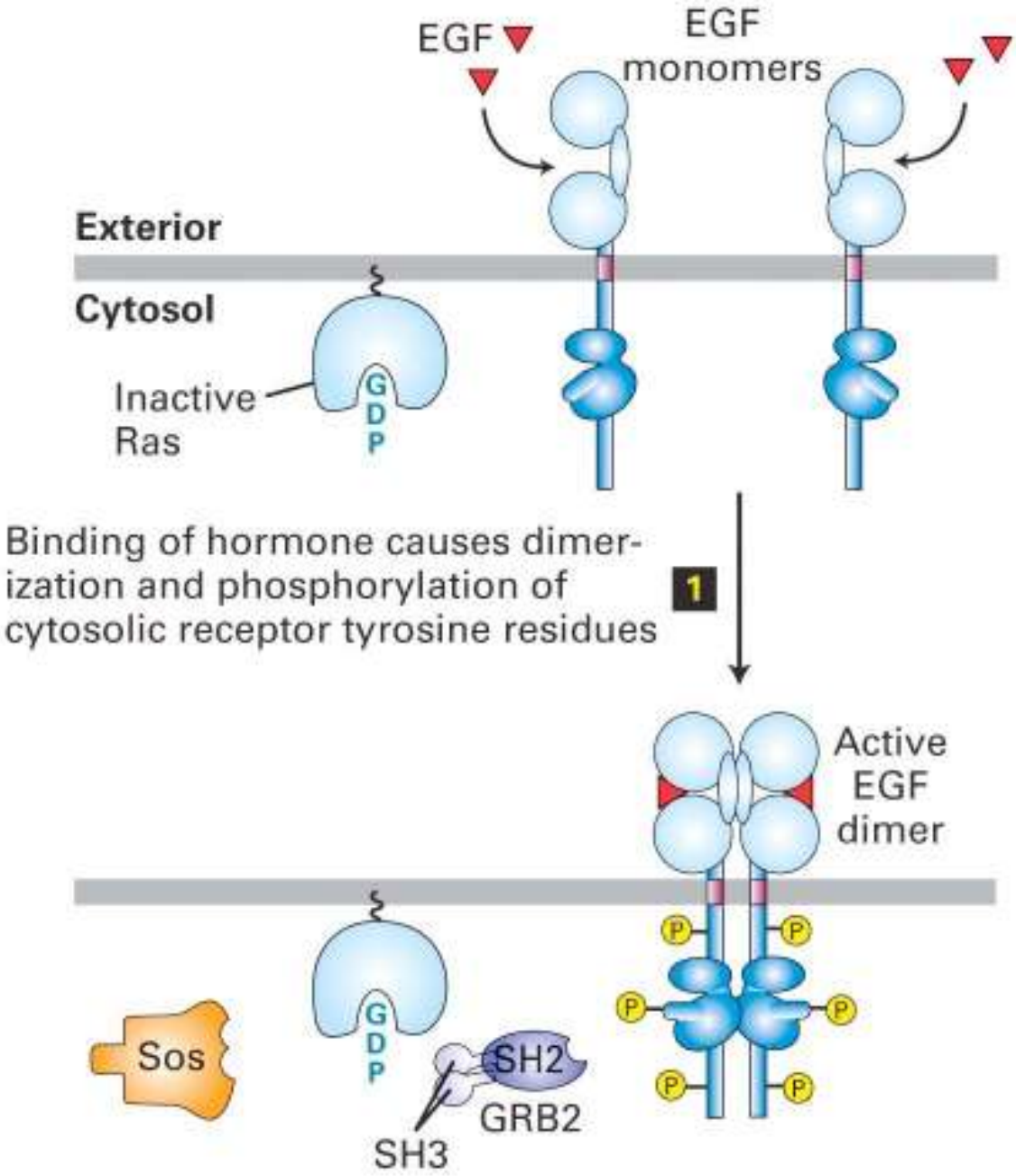
• SH2 protein that binds to phosphorylated RTKs
• It has three domains, one binds the RTK, the other binds Sos

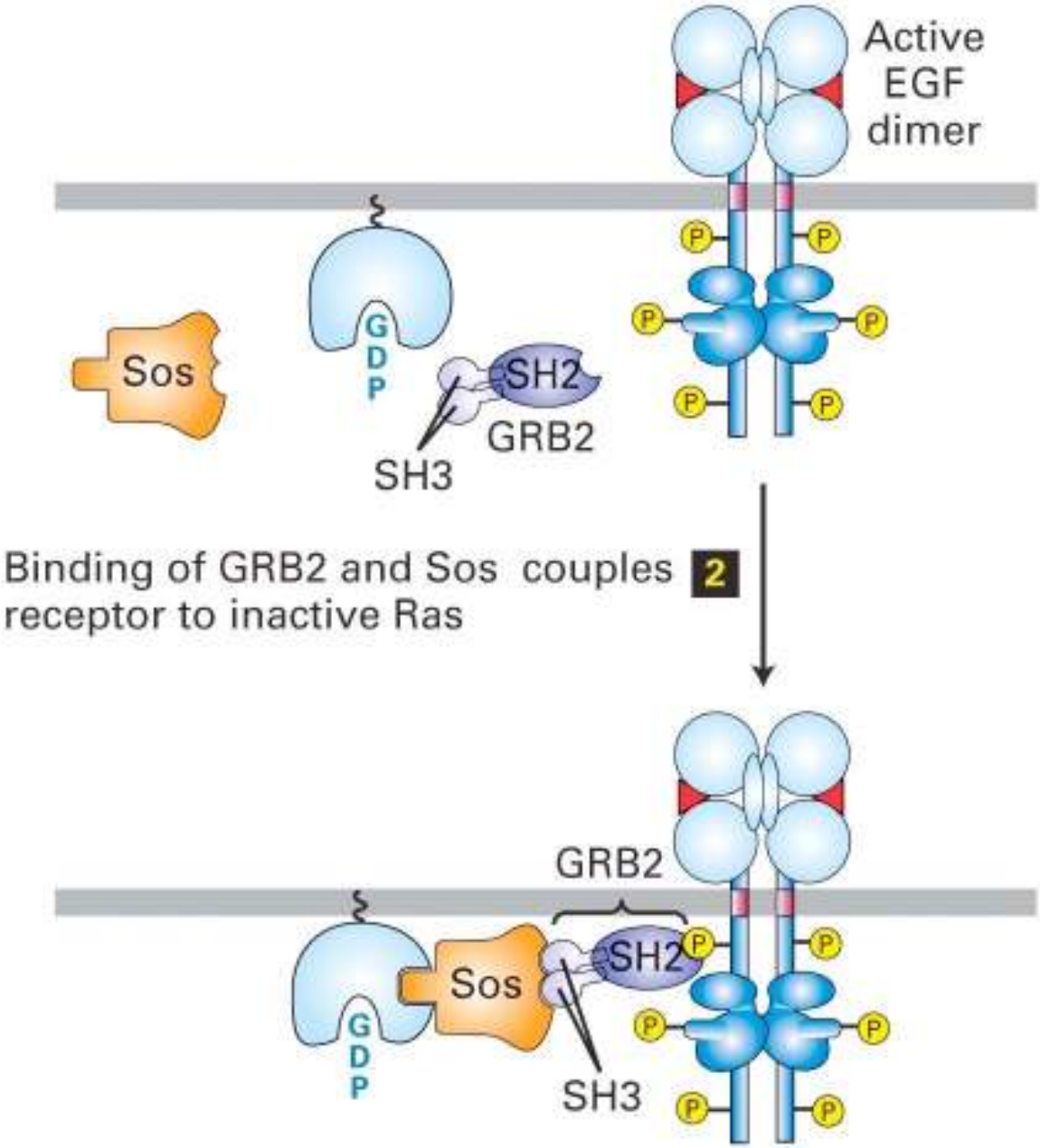
Sos

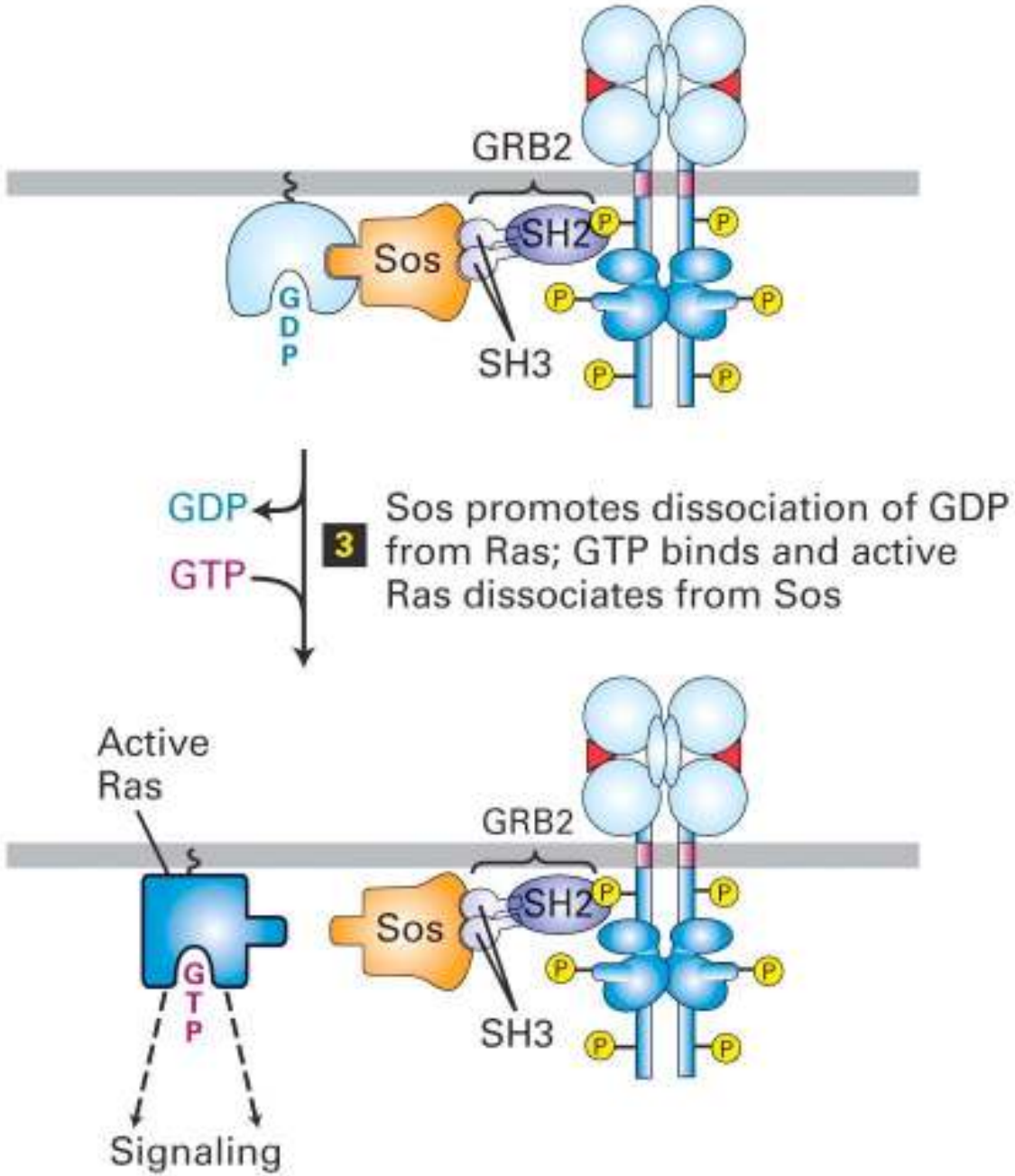
• It is a Ras-GEF. When recruited to the membrane it activates Ras

Raf

• Ras-GTP recruits Raf, which becomes activated as a protein kinase and initiates the MAP kinase cascade

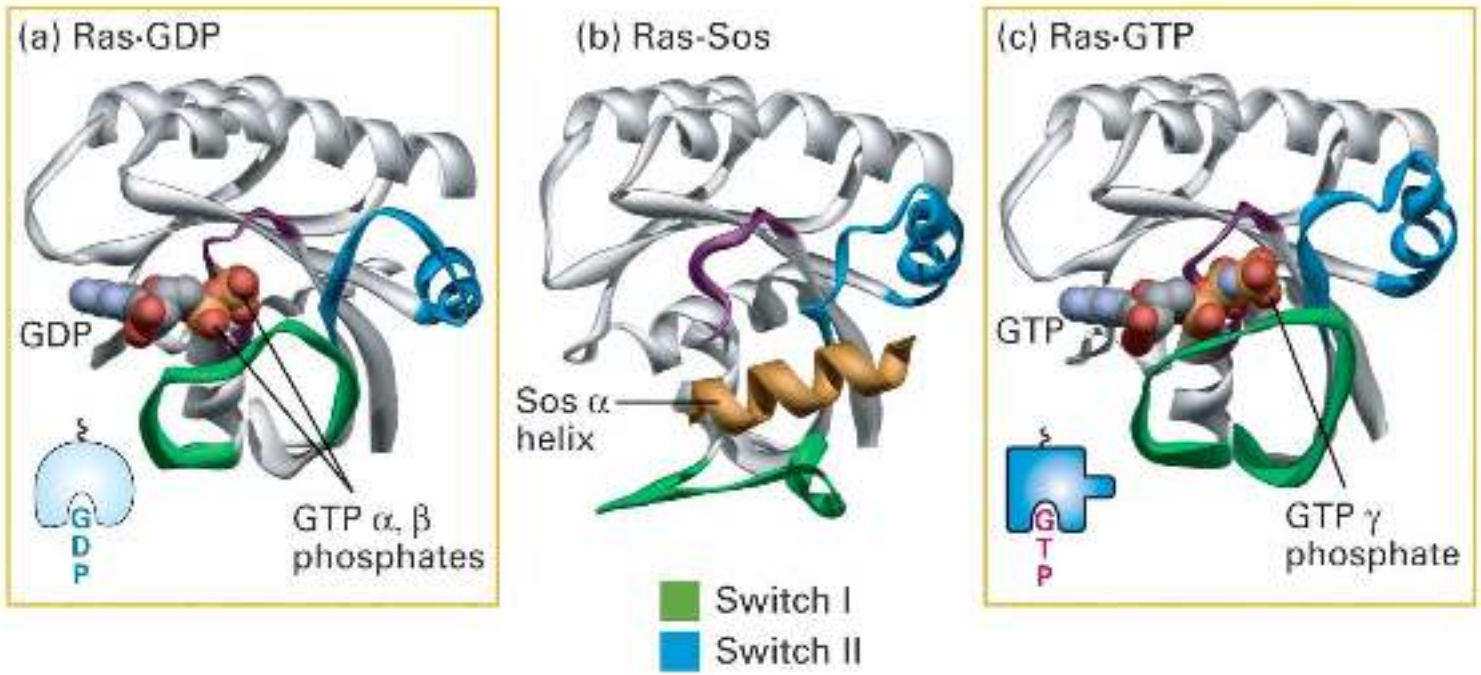

EGF
EGF monomers
Exterior
Cytosol
Inactive Ras
GDP
Binding of hormone causes dimerization and phosphorylation of cytosolic receptor tyrosine residues
1
Active EGF dimer
P
Sos
GDP
SH2
GRB2
SH3


Active
EGF
dimer
Sos
GDP
SH2
GRB2
SH3
Binding of GRB2 and Sos couples receptor to inactive Ras
2
GRB2
Sos
SH2
GDP
SH3

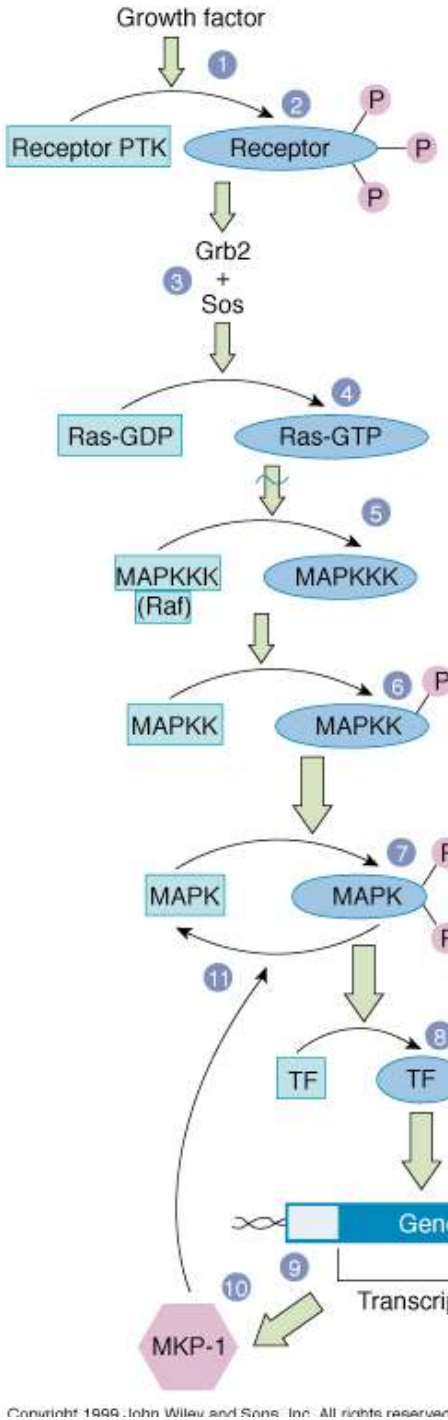

GRB2
SH2
Sos
G
D
P
SH3
GDP
GTP
3
Sos promotes dissociation of GDP from Ras; GTP binds and active Ras dissociates from Sos
Active Ras
GRB2
SH2
Sos
G
T
P
SH3
Signaling

Ras

The experimental support for placing Ras down stream from RTKs in the signaling pathway was obtained in studies of cultured fibroblasm responding to growth factors. When antibodies against Ras were microinjected into these cells they failed to proliferate in the presence of growth factors. Microinjection of a constitutively active Ras mutant lacking GTPase activity resulted in proliferation, even in the absence of growth factors.
Additional studies show that addition of growth factors leads to a rapid increase in the proportion of Ras that is bound to GTP.



Both biochemical and X-ray crystallographic studies have shed light on the mechanism by which Sos acts as an exchange factor for Ras. Binding of Sos to Ras changes the conformation of this protein and opens the nucleotide binding site, resulting in GDP release (a). Binding of the abundant GTP changes the conformation of Ras and displaces Sos (b). The switch I and II regions are those most affected in conformation by the nucleotide state.



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MAP kinase cascade

(Mitogen-Activated Protein: activated by a mitosis-stimulating growth factor)

Recruitment of Raf by activated Ras to the plasma membrane activates it as a kinase (MAPKKK)

Raf phosphorylates MAPKK

MAPKK phosphorylates MAPK

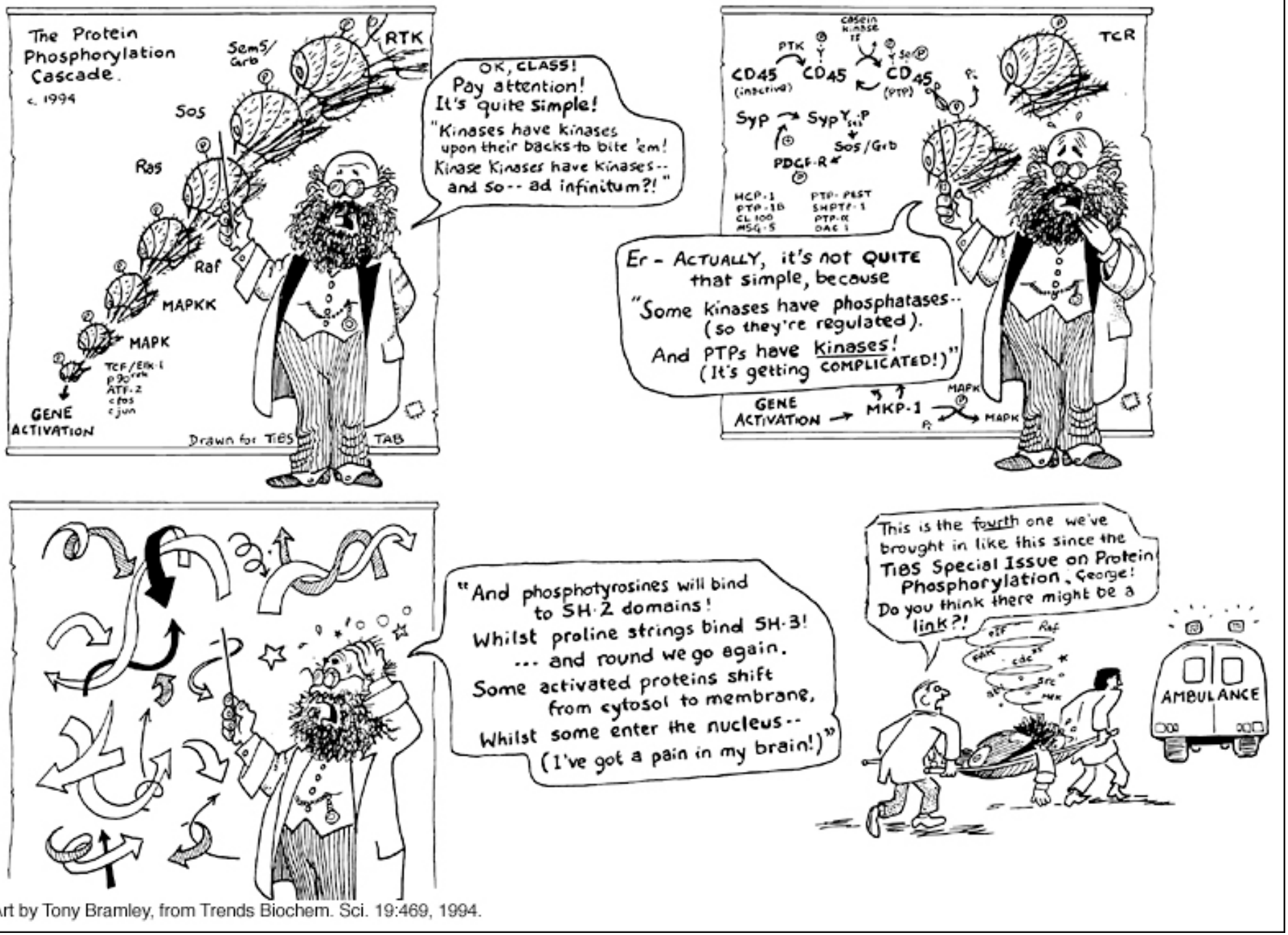
MAPK phosphorylates transcription factors like Elk-1

Elk-1 activates the transcription of Fos and June. These proteins then make a dimer that activates the transcription of cell proliferating genes

The transcription of MAPK phosphatase (MKP-1) is also activated. MKP-1 dephosphorylates MAPK and stop signalling

This pathway is generally used in eukaryotes for many different functions. Different types of information are transmitted thanks to the existence of different isoforms for each of the cascade proteins.

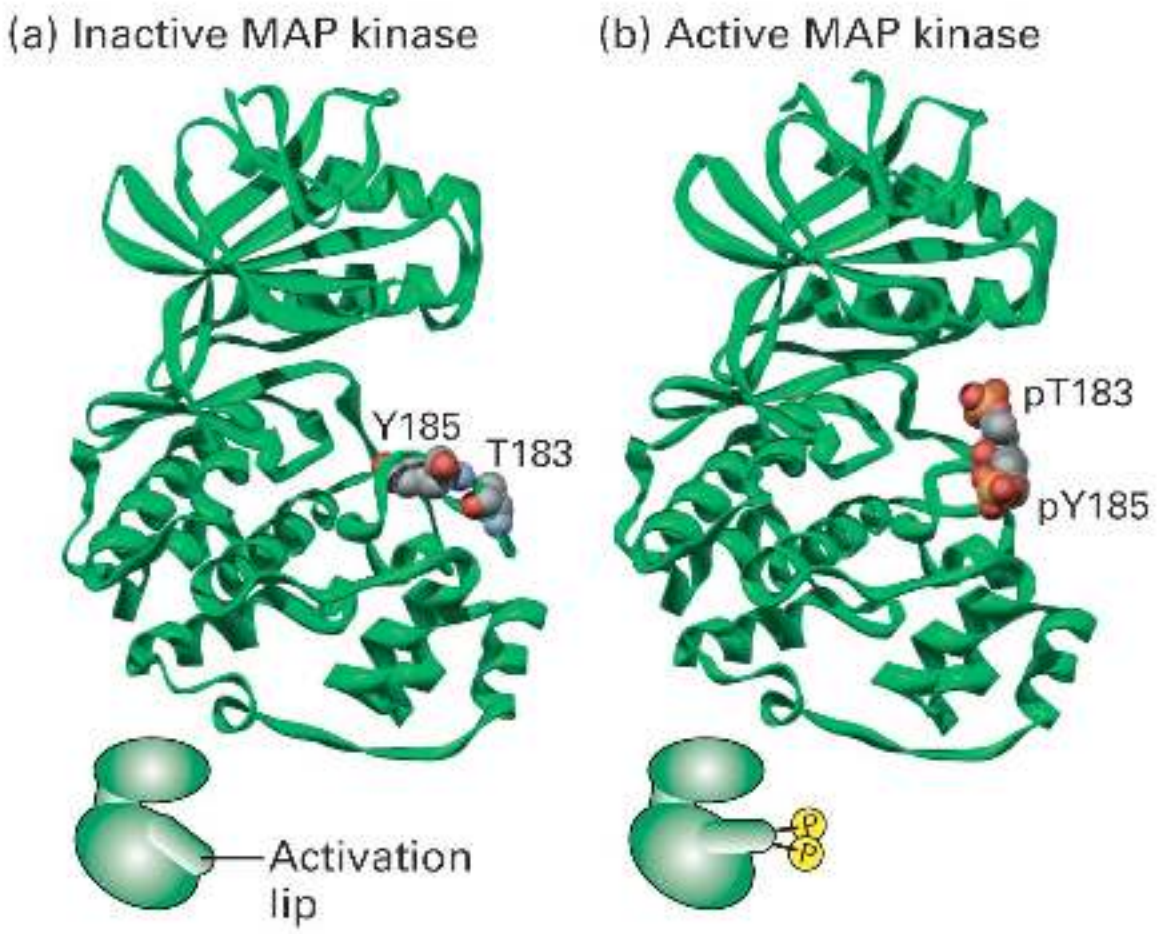
The human genome encodes for 2000 kinases and 1000 phosphatases



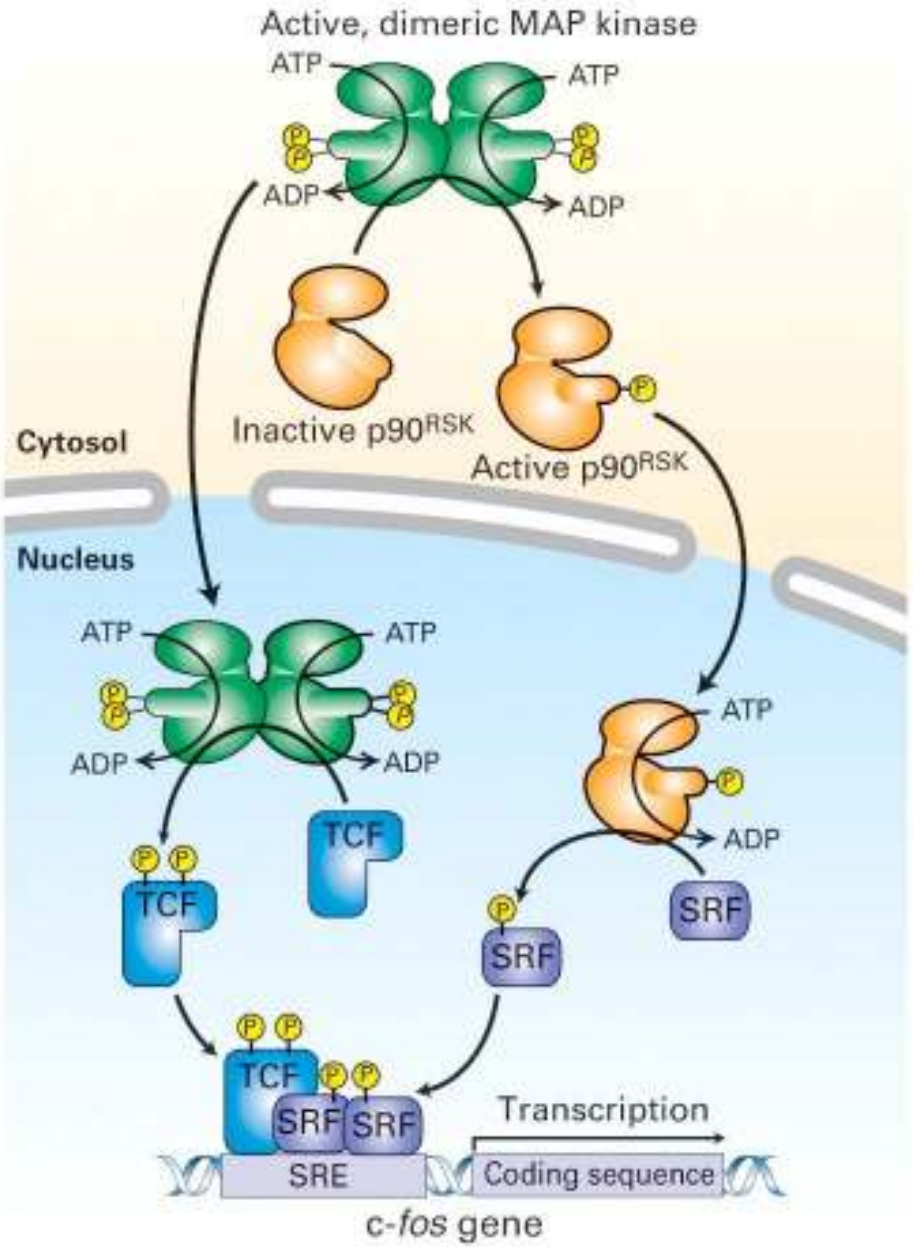
Art by Tony Bramley, from Trends Biochem. Sci. 19:469, 1994.

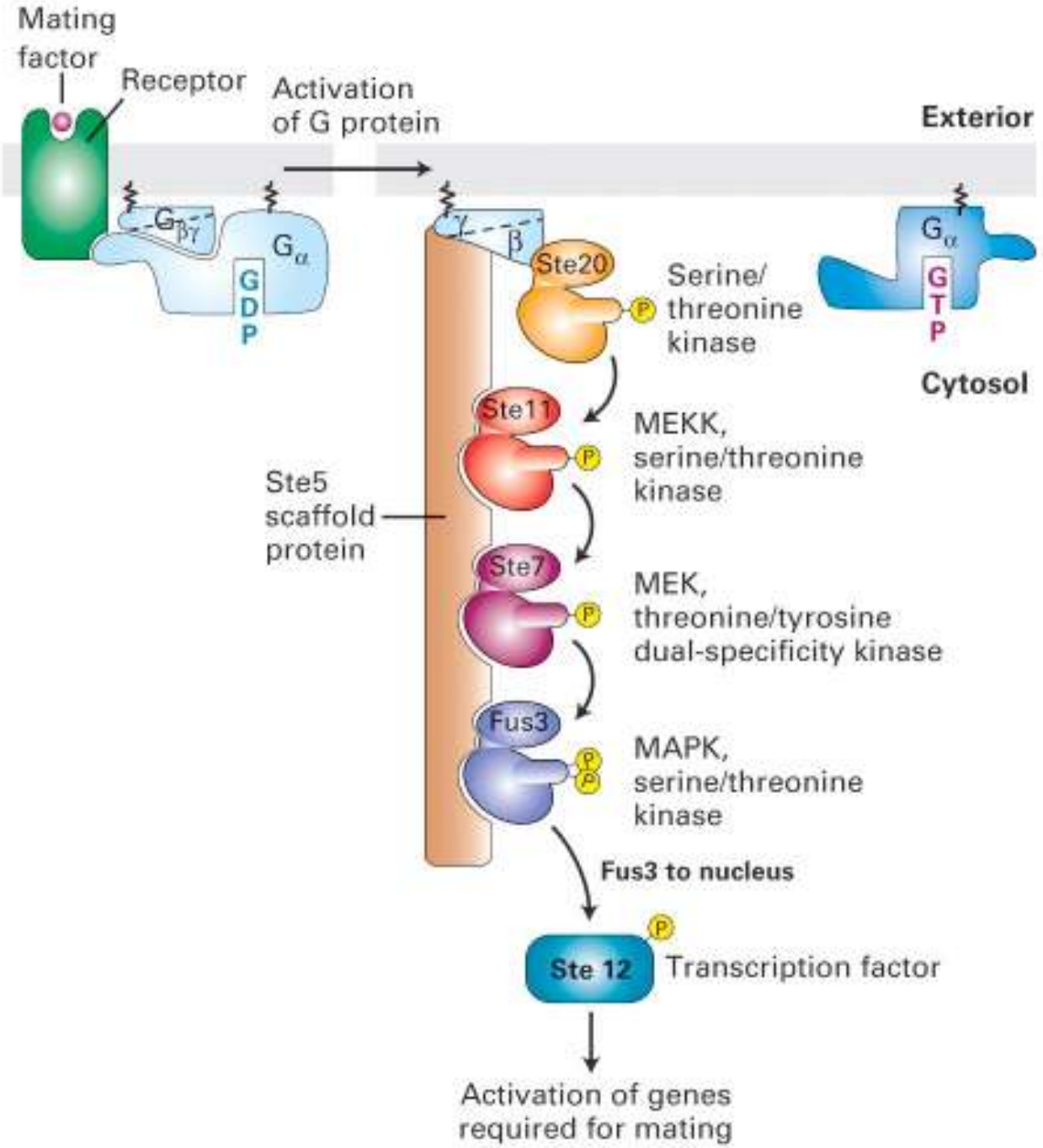
MAP Kinase

In its unphosphorylated state MAPK is inactive, its catalytic site blocked by the "phosphorylation lip" (a) . Binding of MAPKK (MEK) to MAPK and phosphorylation of a Tyr and a Thr in the phosphorylation lip alters the conformation of the lip, allowing the binding of ATP to the catalytic site (b).



The phosphotyrosine also contributes to the binding of MAPK substrates. In addition, the phosphorylation of the two residues in the lip region promotes dimerization of MAPK. This dimeric form can translocate into the nucleus where it regulates the activity of transcription factors.


Active, dimeric MAP kinase
ATP
ATP
ADP
ADP
Inactive p90RSK
Active p90RSK
Cytosol
Nucleus
ATP
ATP
ADP
ADP
TCF
TCF
ATP
ADP
SRF
SRF
TCF
SRF
SRF
Transcription
SRE
Coding sequence
c-fos gene


Mating factor
Receptor
Activation of G protein
Exterior
Cytosol
Ste20
Serine/ threonine kinase
Ste11
MEKK, serine/threonine kinase
Ste5 scaffold protein
Ste7
MEK, threonine/tyrosine dual-specificity kinase
Fus3
MAPK, serine/threonine kinase
Fus3 to nucleus
Ste 12
Transcription factor
Activation of genes required for mating

IV Integrin Signaling

• Normal cells cannot grow in suspension (as cancer cells do), but need to attach to a surface that resembles the extracellular matrix (ECM) in a tissue.

• Cells have transmembrane proteins called integrins that anchor them to materials in the ECM (fibronectin, collagen, proteoglycans, etc).

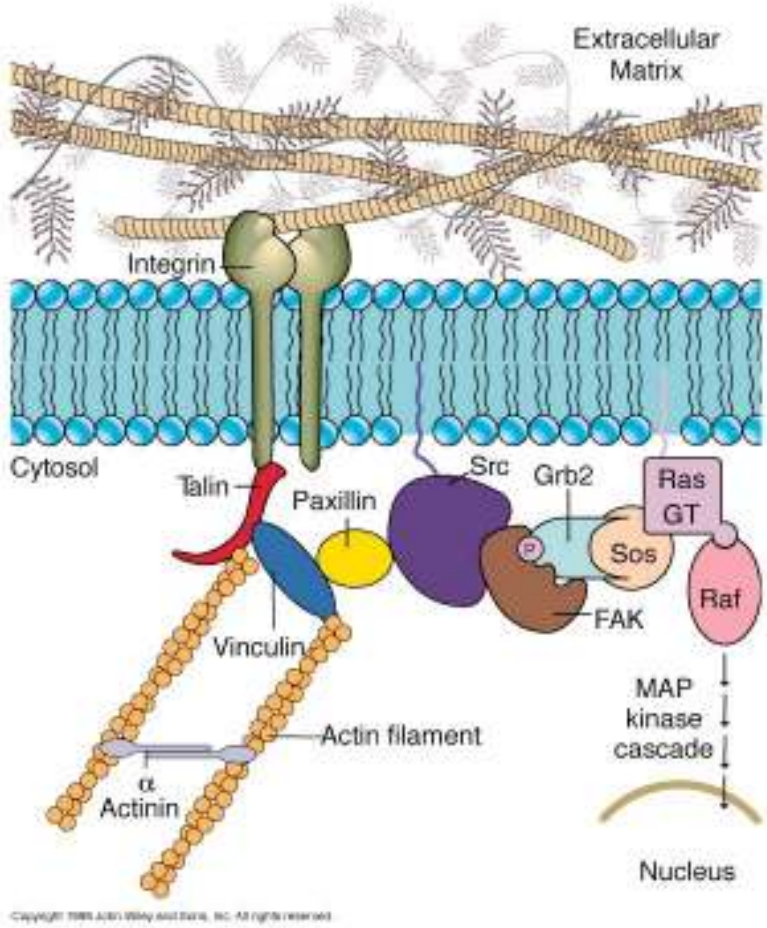
• Interaction of integrins with an extracellular ligand generates a variety of signals, some essential for cell growth and differentiation.

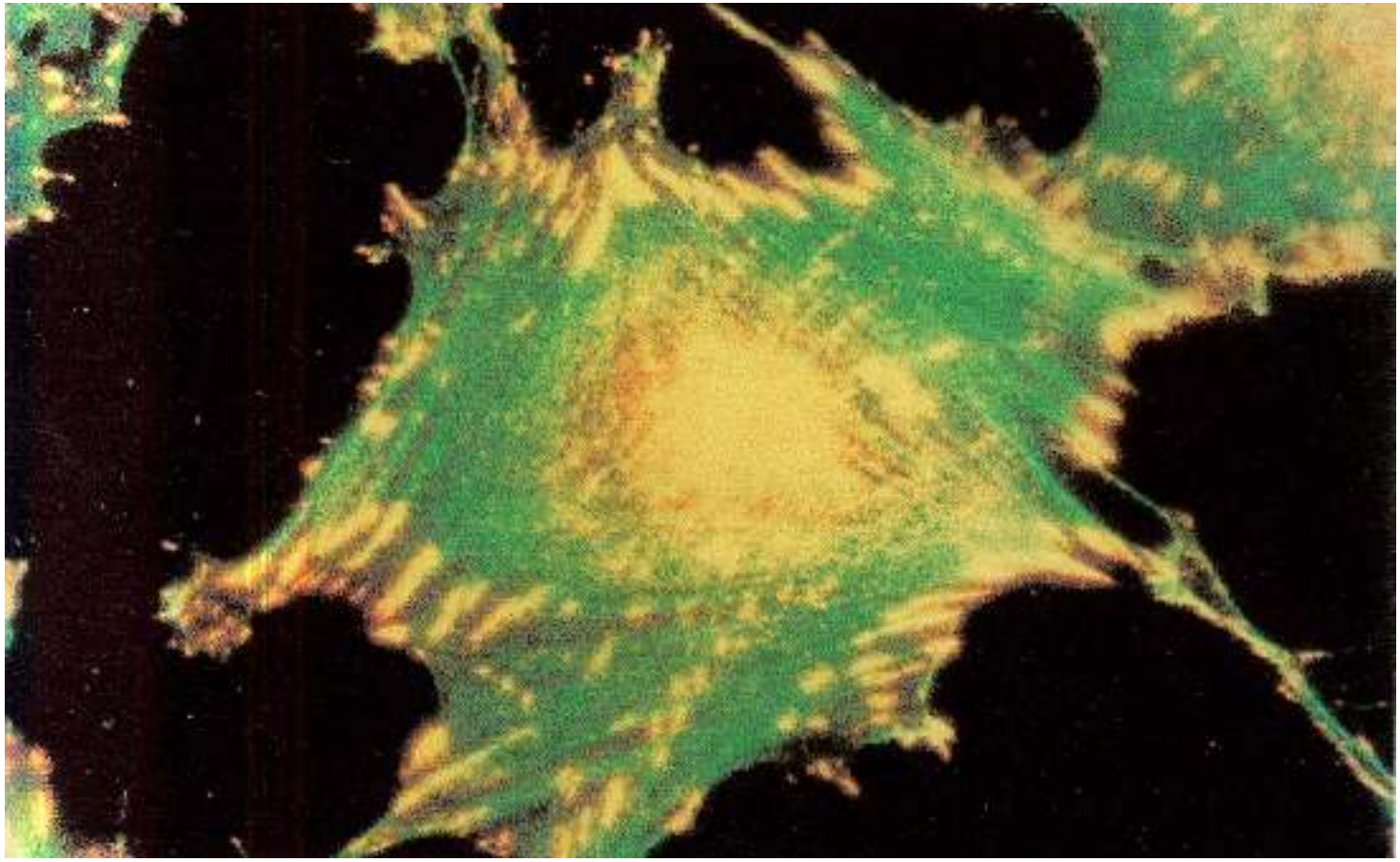
The interaction of the cell with the ECM is done through focal adhesions containing clustered integrins, cytoplasmic proteins and actin stress fibers

The tyrosine kinase Src is localized to focal adhesions.

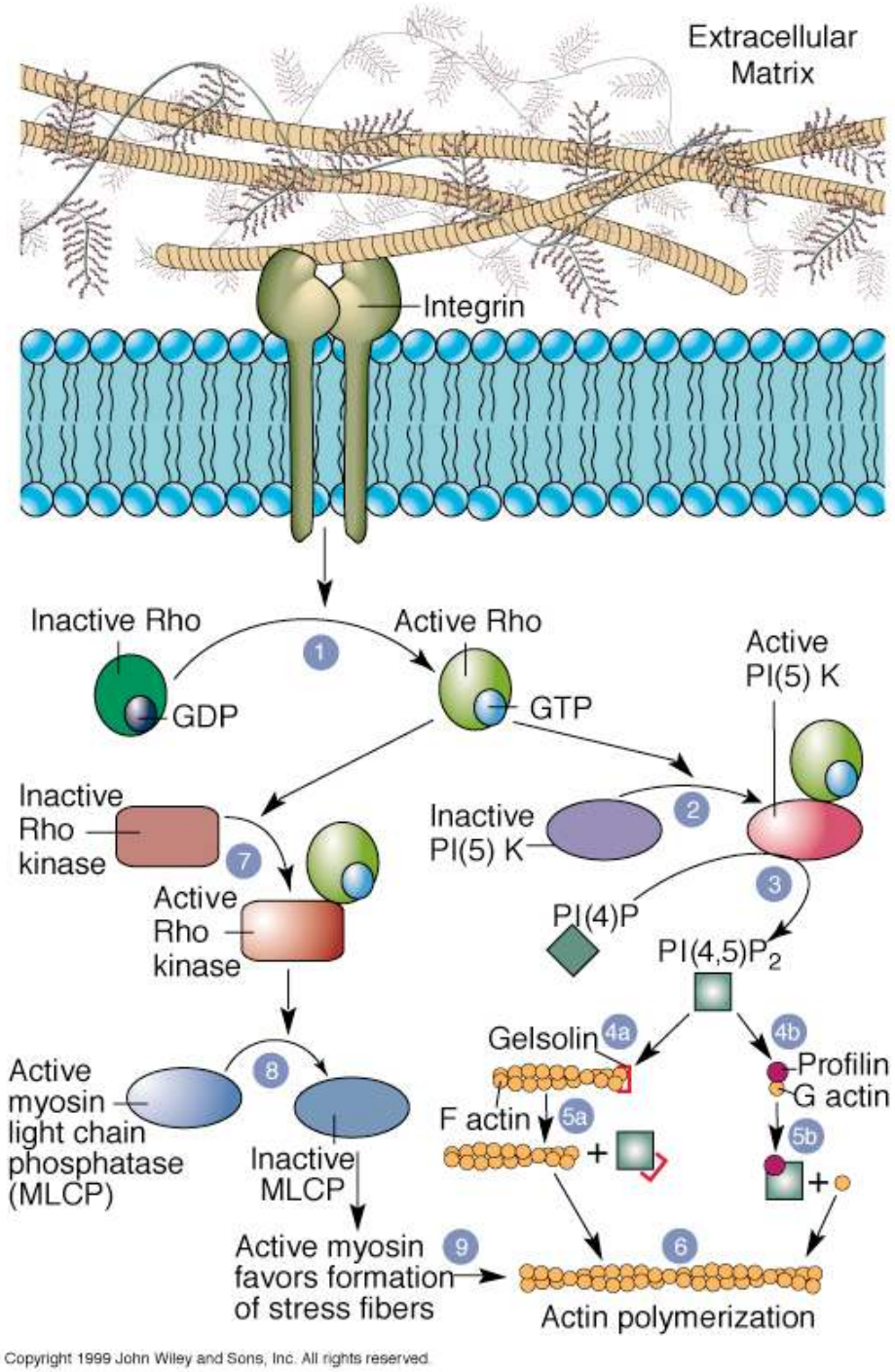
Src phosphorylates the focal adhesion kinase (FAK)

Phosphorylated FAK is bound by SH2 proteins like Grb2-Sos, activating the MAP kinase cascade





Activation of FAK also results in signals that affect ribosomal components resulting in the regulation of protein synthesis required for the transition from G1 to S phase



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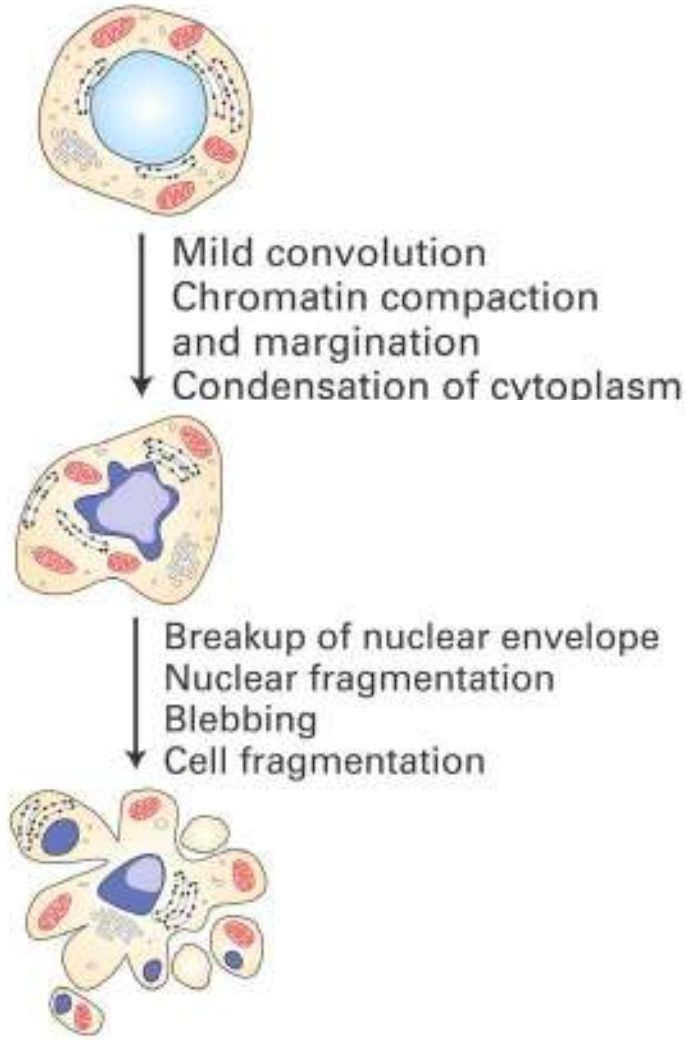
The assembly of focal adhesions has a large effect in the organization of the actin cytoskeleton in the cell through the G protein Rho.

On one pathway activated Rho activates PIP2 signaling that affects actin binding proteins controlling polymerization.

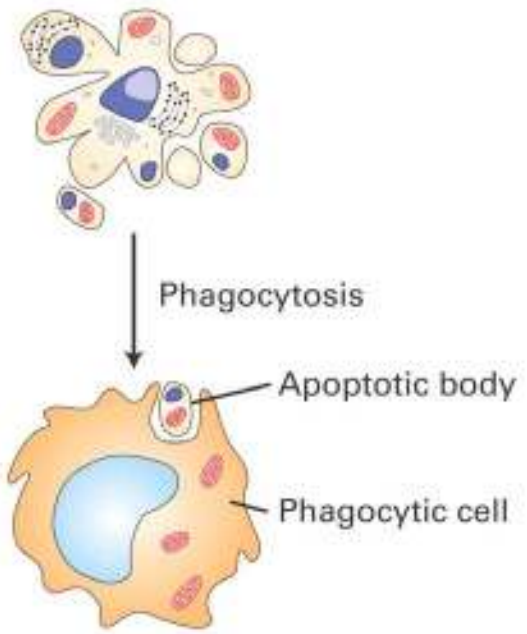
In another pathway Rho activates the Rho kinase that inactivates a myosin light chain phosphatase, leading to the activation of myosin and the organization of actin into stress fibers

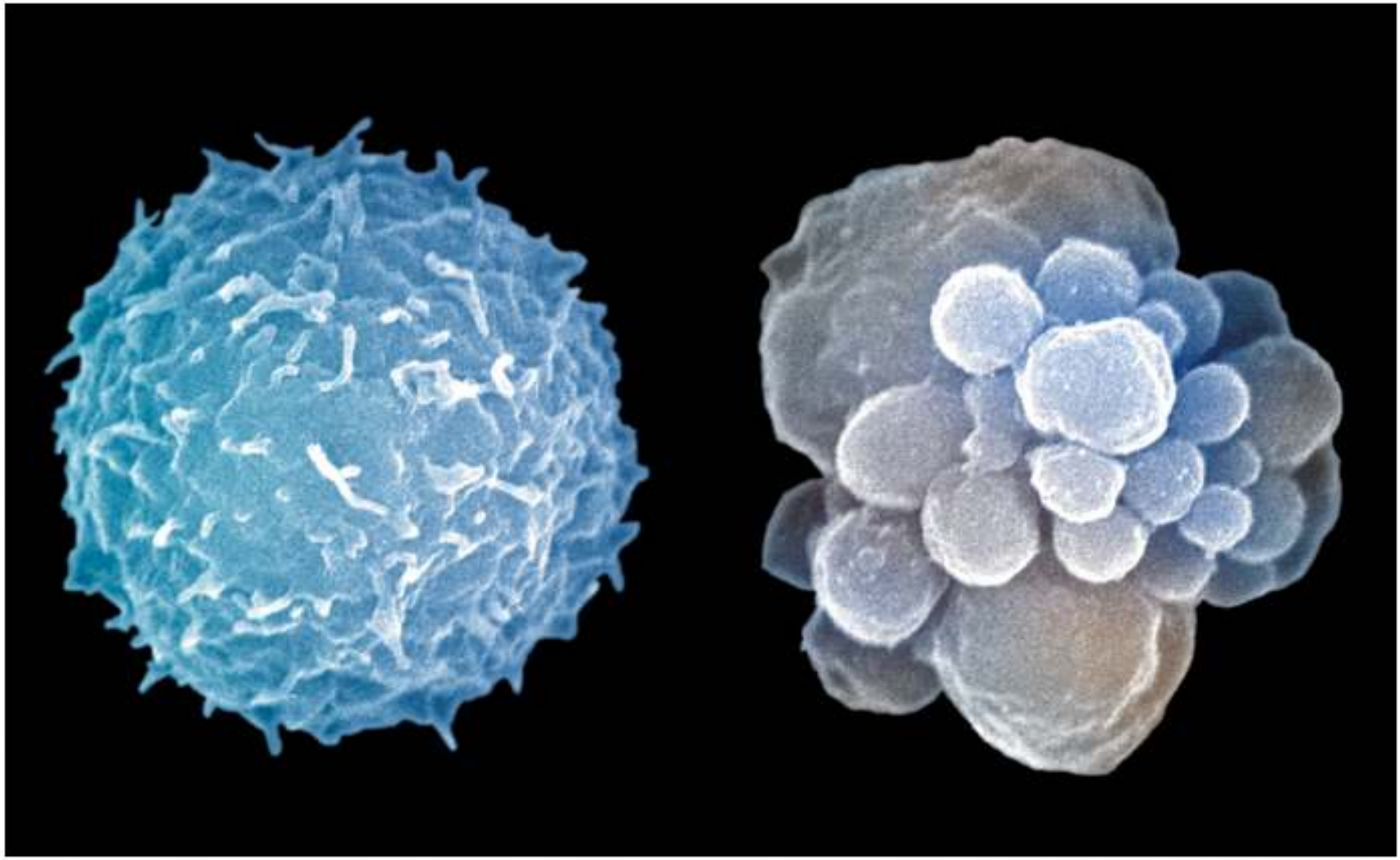
V Cell Signaling and Apoptosis

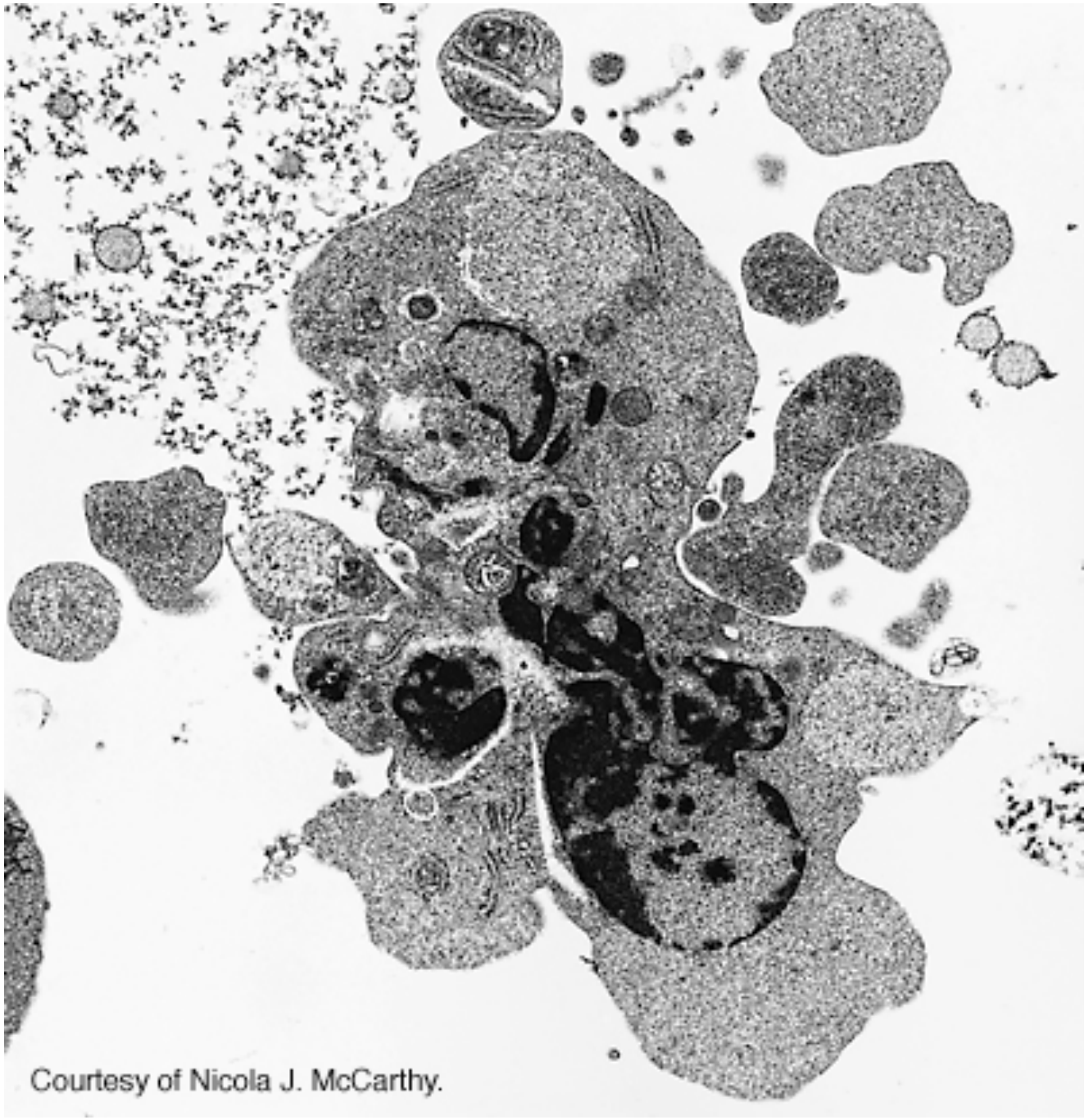


During apoptosis cells die in a regulated, well programmed fashion, following a sequence of morphological changes:

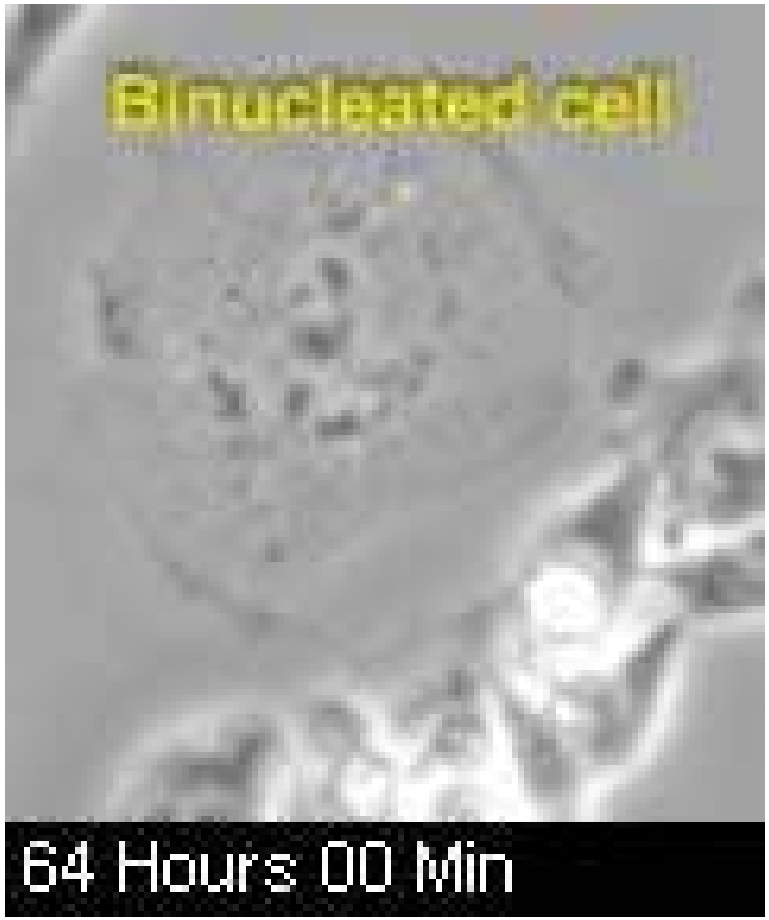
- The nucleus condenses and the DNA fragments
- Lost of adhesion and shrinkage of the cell
- Blebbing
- Engulfment by phagocytosis







Courtesy of Nicola J. McCarthy.

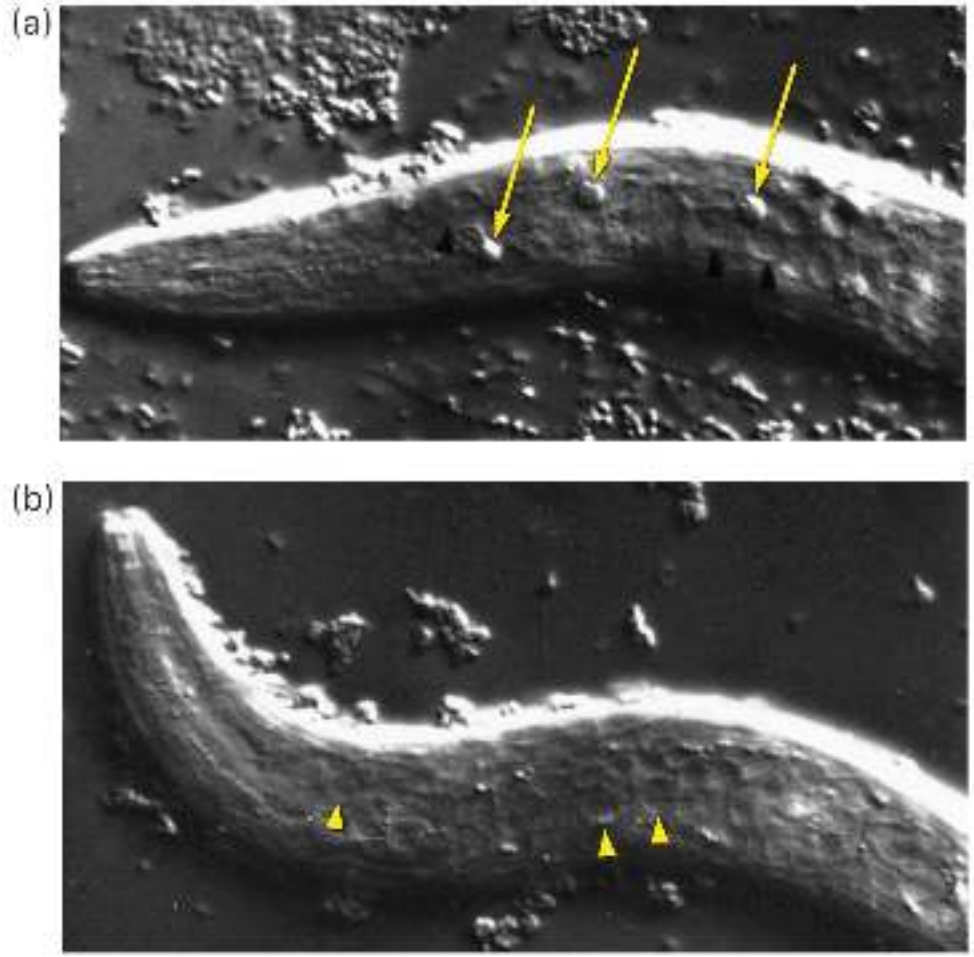

Binucleated cell
64 Hours 00 Min

Importance of Apoptosis

- Billions of cells in our body die by apoptosis every day.

- Apoptosis is essential during development
  - morphogenesis
  - neurons
  - T lymphocytes

- Apoptosis is used as a control against cancer development

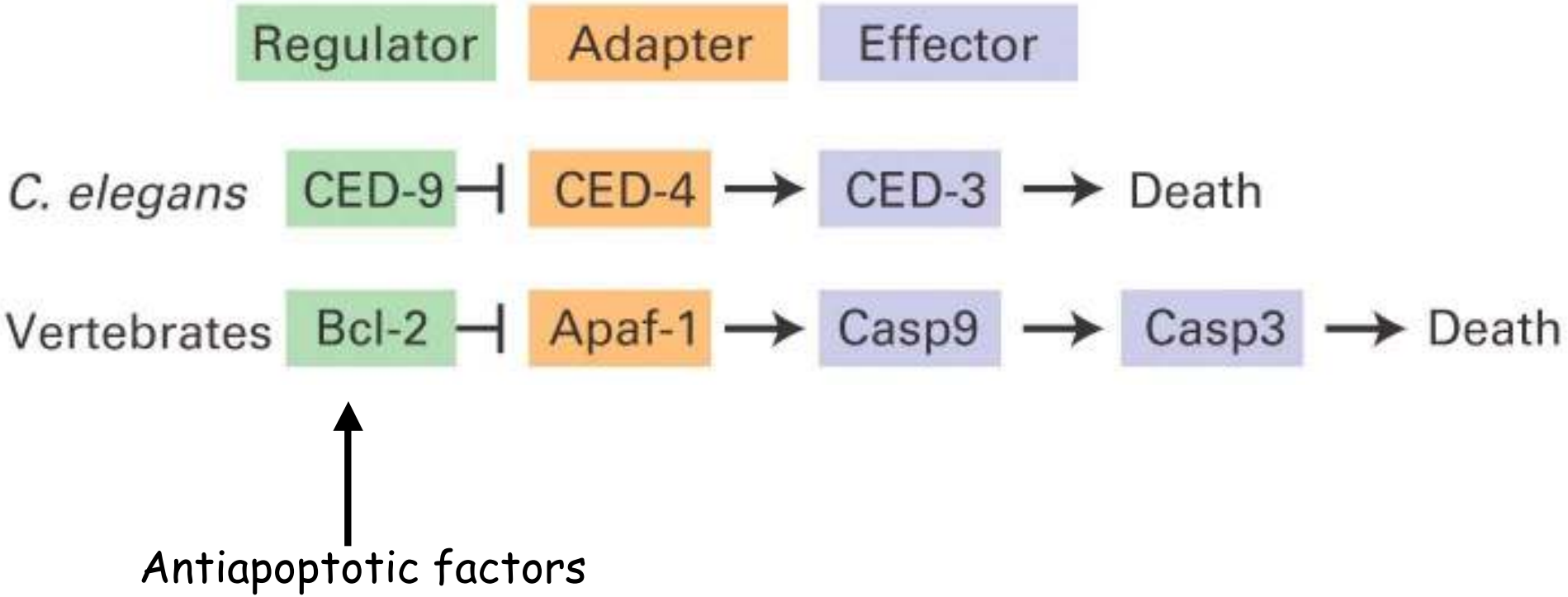


The nematode worm C. Elegans as a model system for apoptosis:

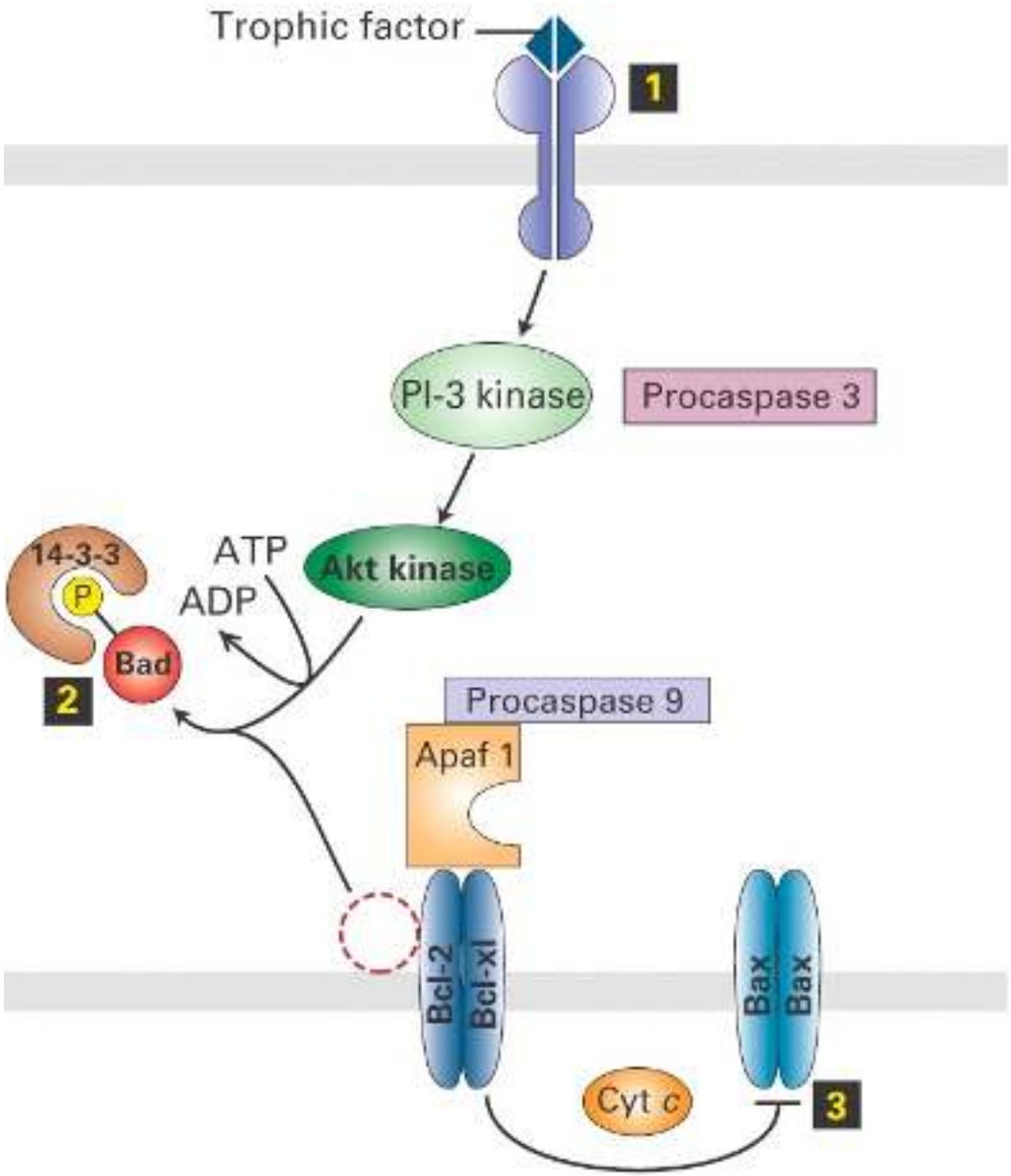
- discovery of the fist **CASPASE**

Cysteine proteases that triggers most of the apoptotic response:

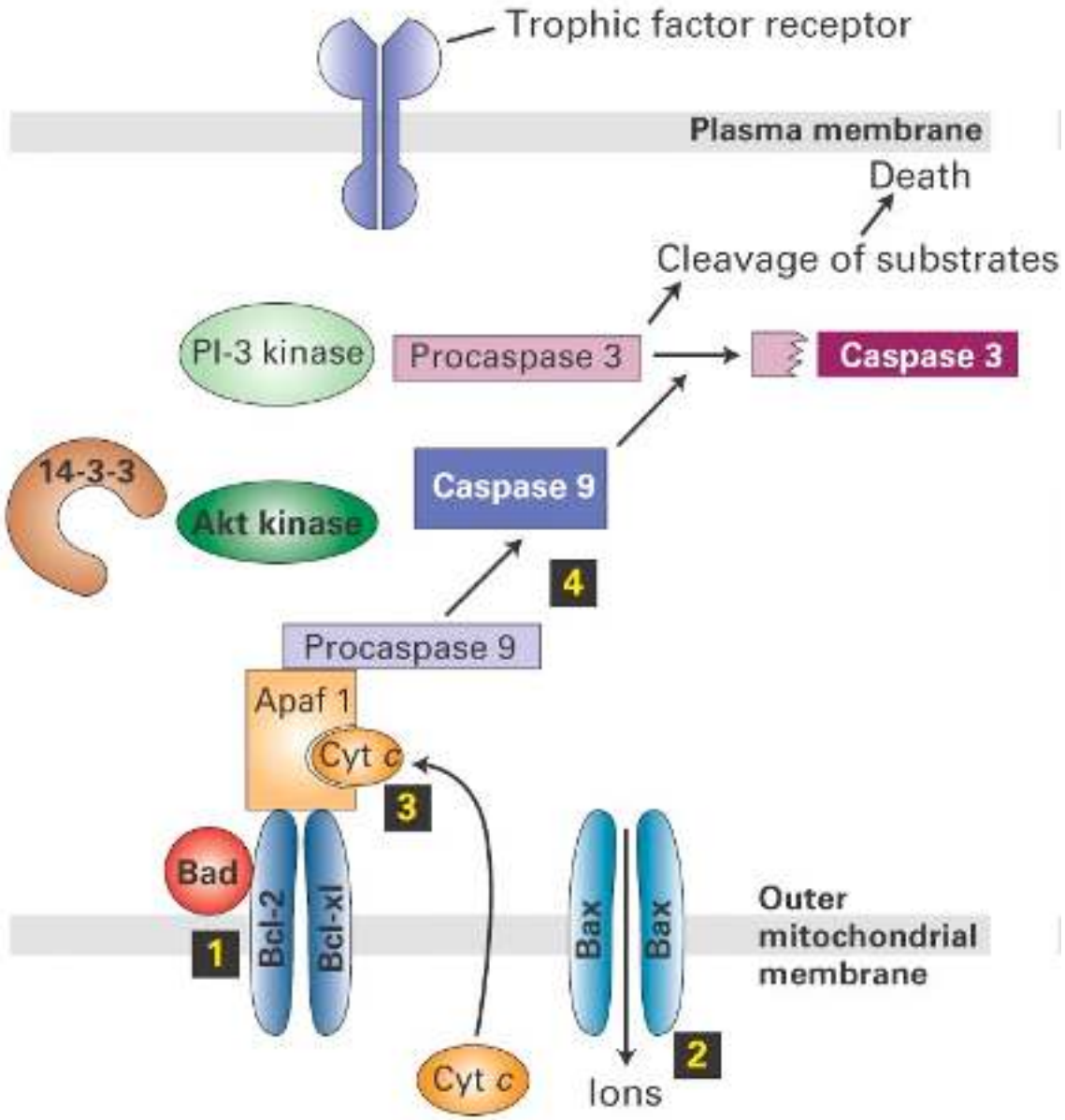
- Kinases
- Nuclear lamins
- Cytoskeleton
- DNA endonuclease


Regulator
Adapter
Effector
C. elegans
CED-9
CED-4
CED-3
Death
Vertebrates
Bcl-2
Apaf-1
Casp9
Casp3
Death
Antiapoptotic factors

Presence of a **trophic factor** is required for survival: **Inhibition of Caspase Activation**

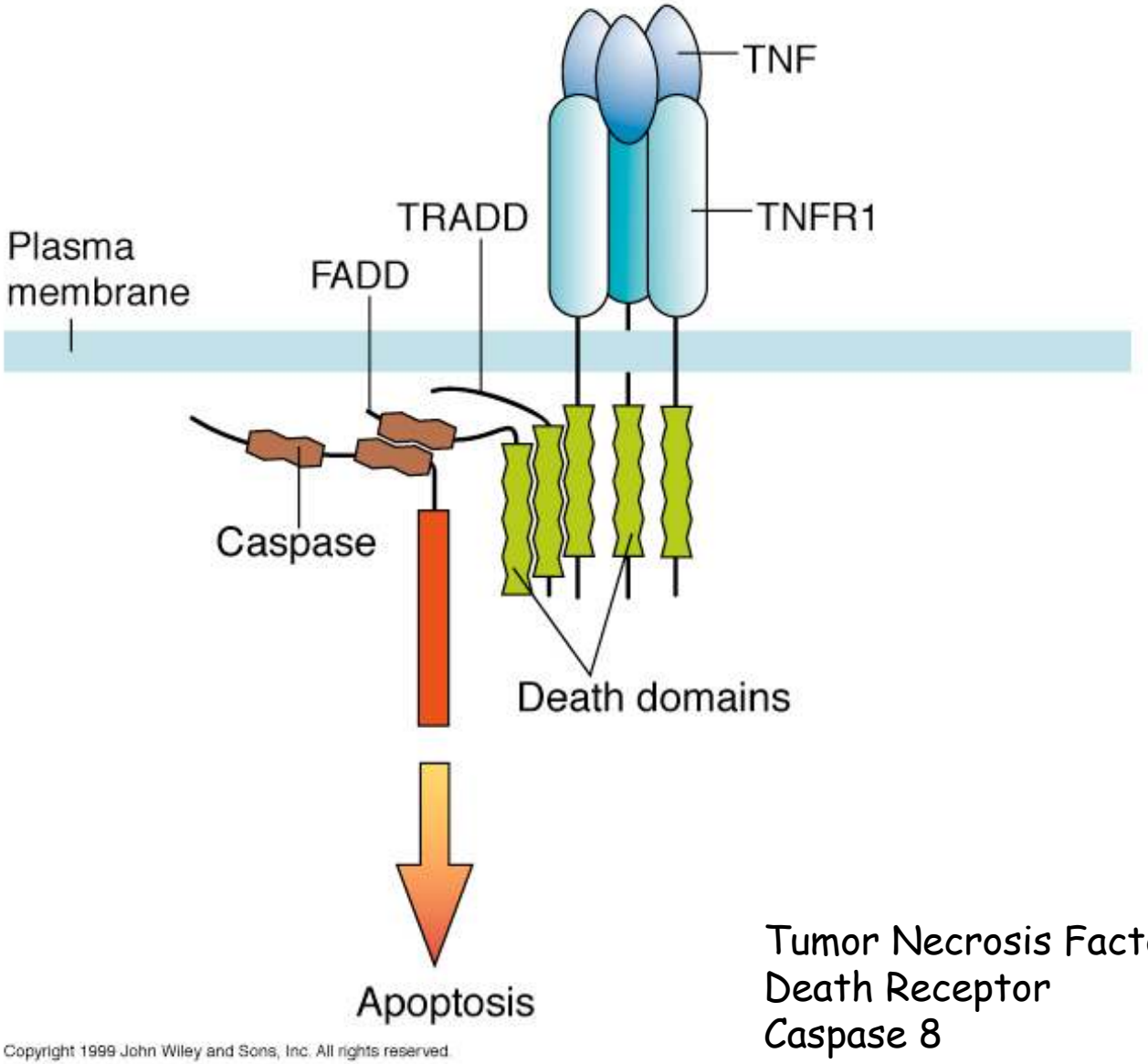


Absence of trophic factor leads to **caspase activation** and apoptosis



MOVIE

Death Signals activation of Caspases



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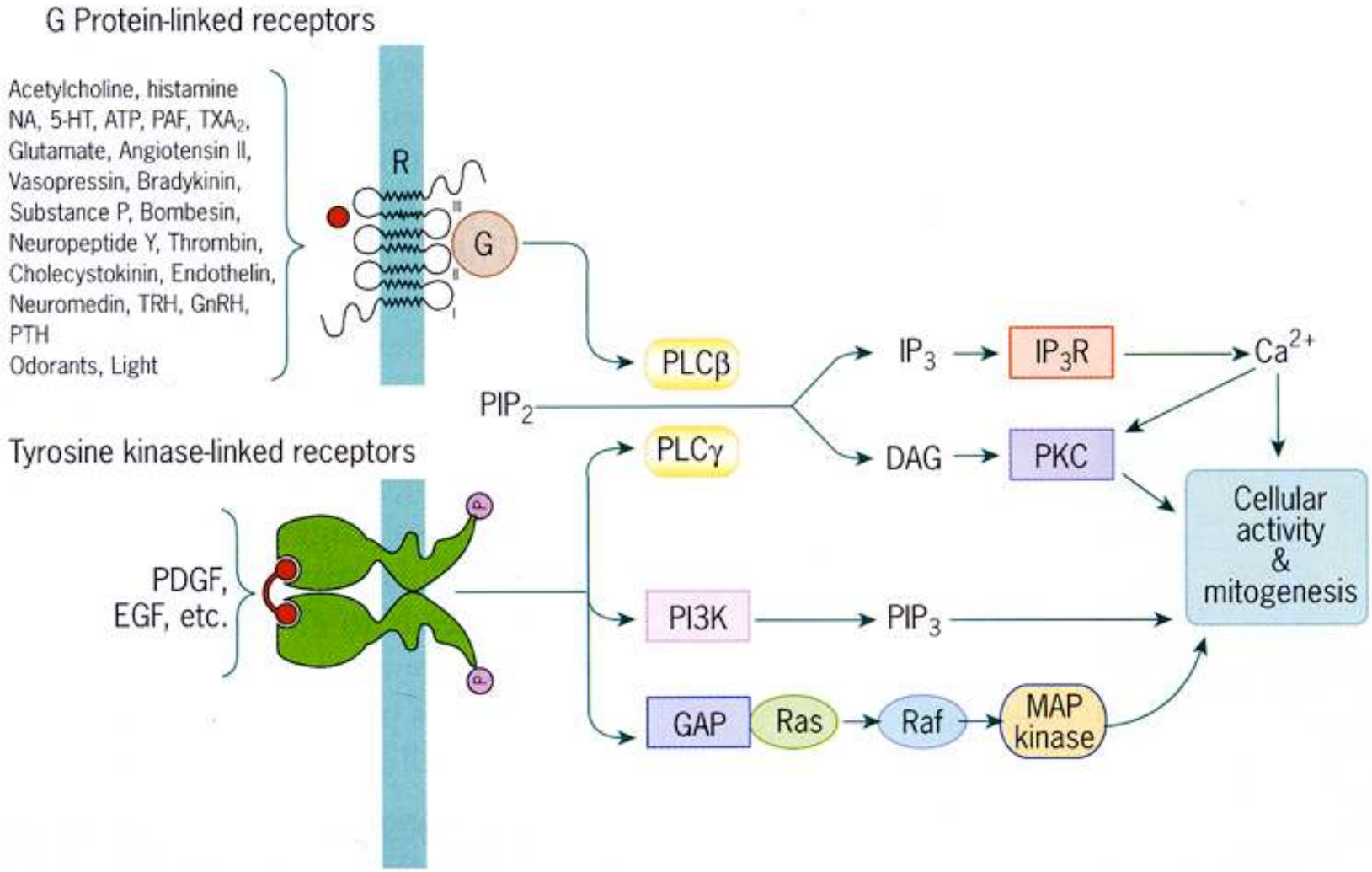
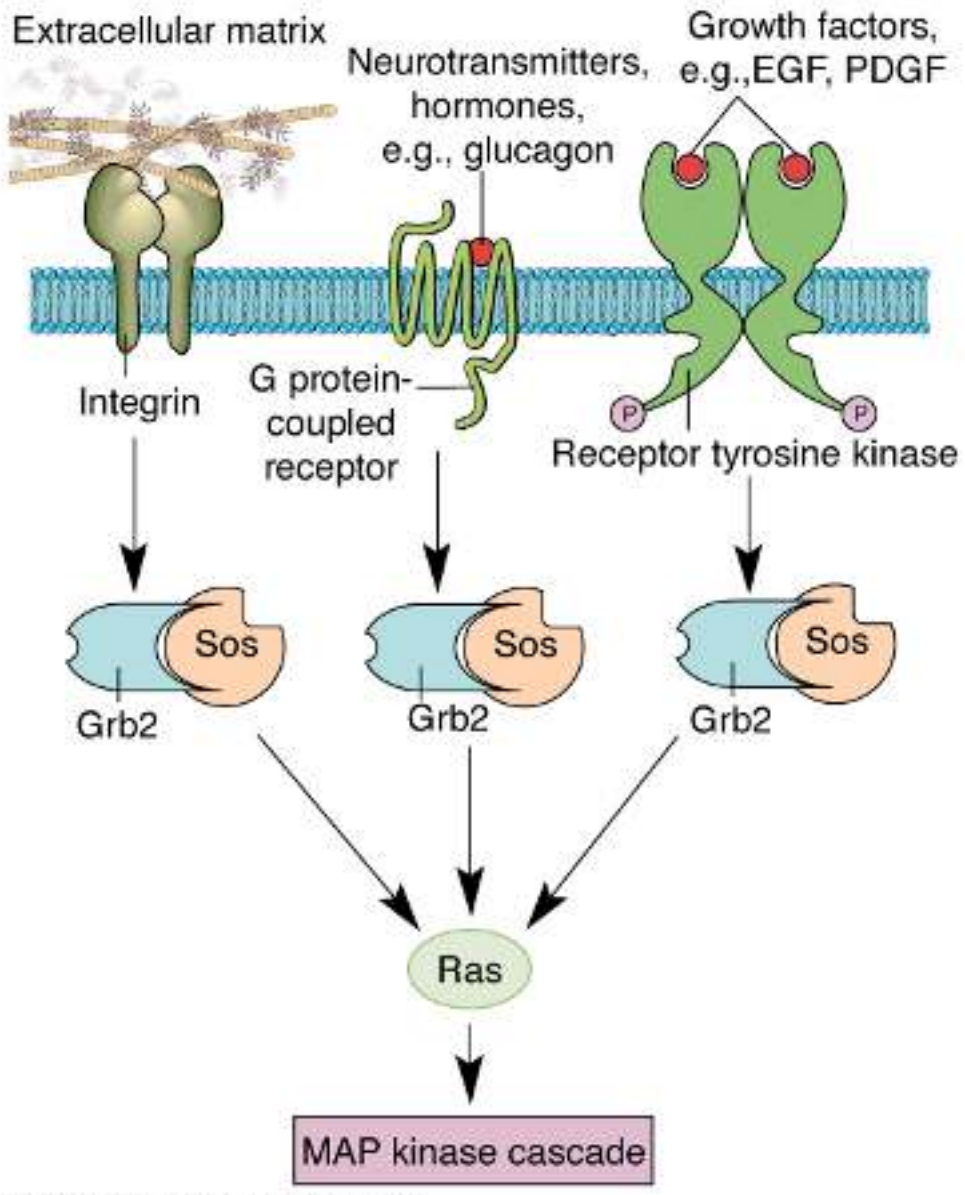
Tumor Necrosis Factor
Death Receptor
Caspase 8

VI Relationship between Signaling Pathways

A single cell has dozens of different receptors sending signals to the inside of the cell simultaneously. These signals, along with different pathways, are integrated in the cell to give rise to a coordinated response. Signaling pathways can relate to each other in different ways:





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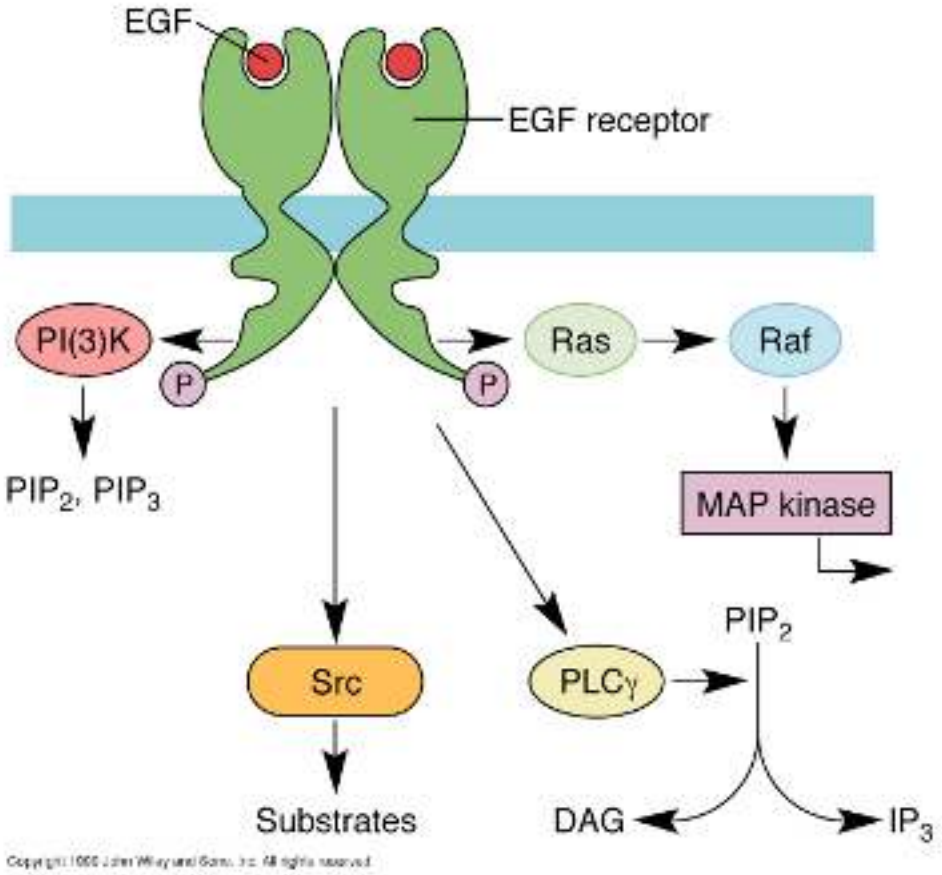
Converging Signaling Pathways

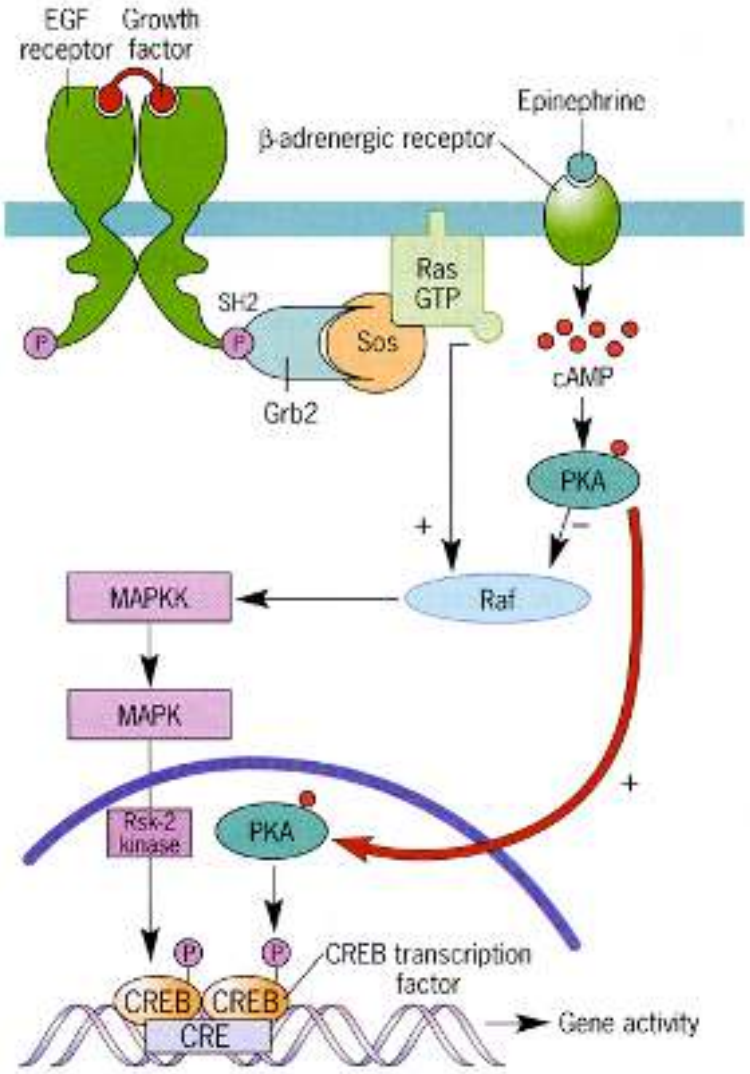
G protein-coupled receptors, RTKs and integrins can all relay signals that result in the recruitment to the membrane of the adaptor protein Grb2 and the activation of Ras and the MAP kinase cascade.

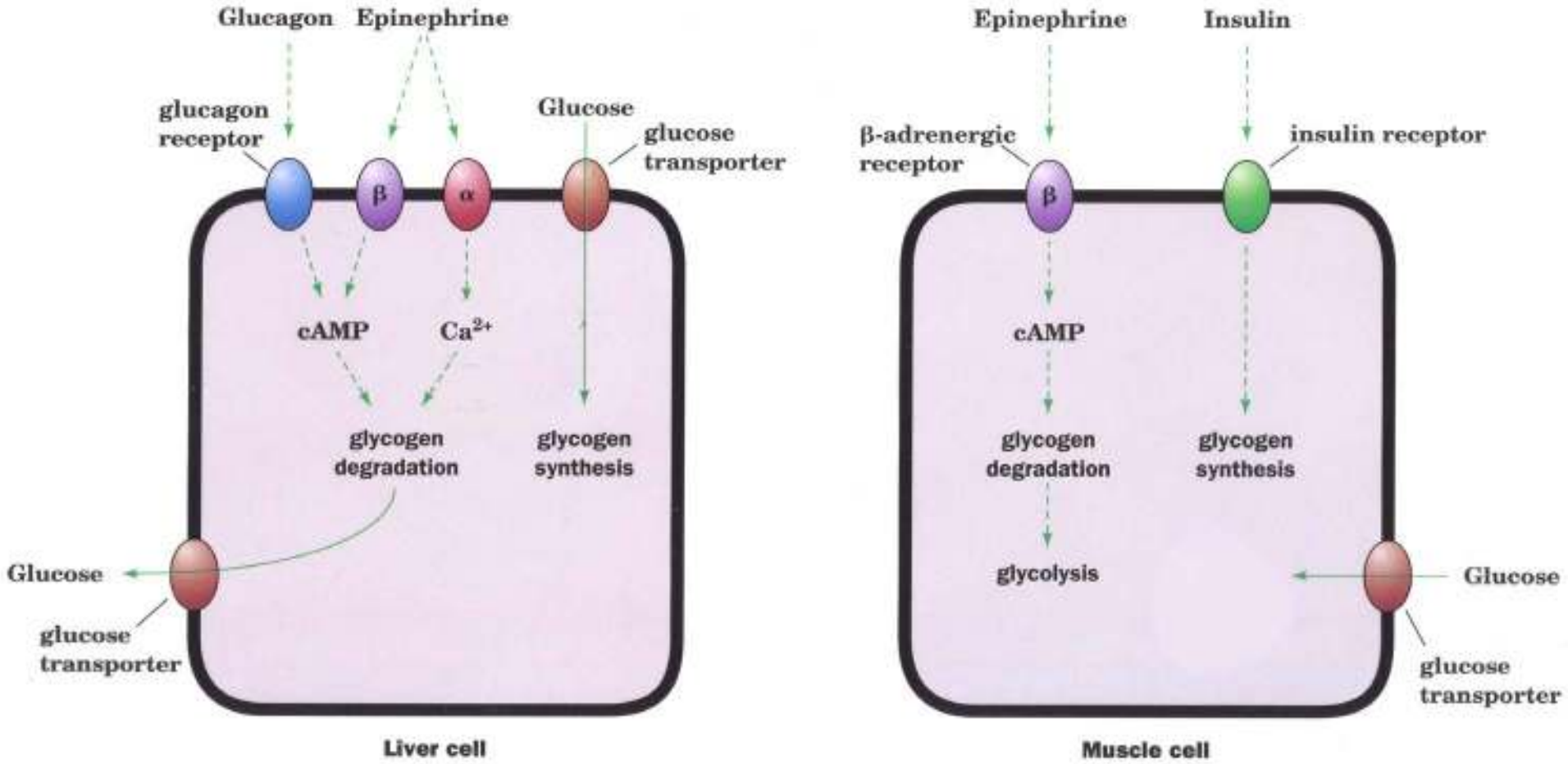
Diverging Signaling Pathways

Activation of the EGF RTK generates phosphotyrosine sites that interact with a variety of SH2 proteins, thus sending a signal down several pathways



Crosstalk between Signaling Pathways



EGF/epinephrine

Hormone Responses in Glucose Metabolism: liver and muscle cells