Dissertation

Faculty of Biology/Chemistry at the University of Kassel (GHK)

Molecular Analysis of the Growth-Differentiation-Transition in *Dictyostelium*

by

Changjiang Zeng

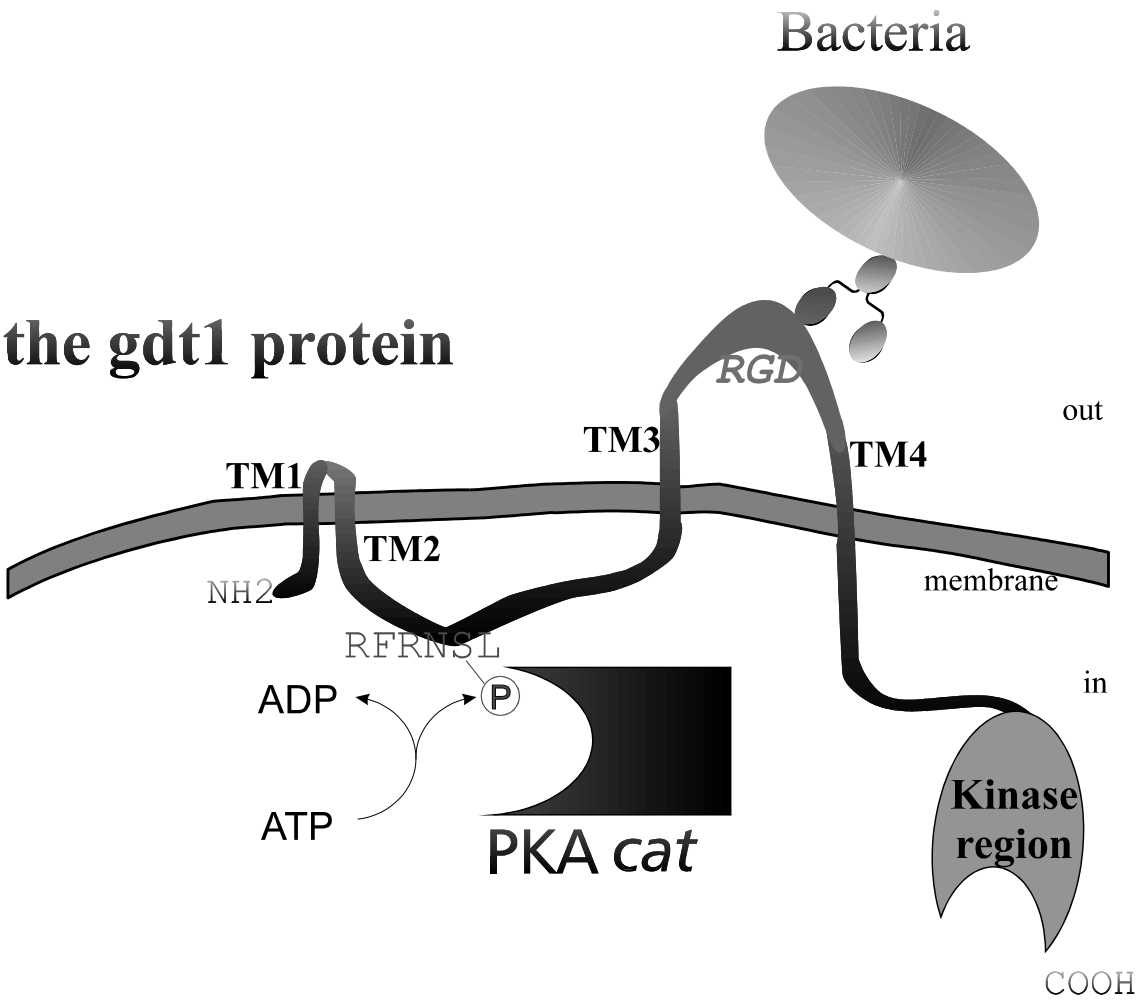

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TABLE OF CONTENTS

ABBREVIATIONS

AC	adenylyl cyclase
AP	alkaline phosphatase
ATP	adenosine 5'-triphosphate
Ab	antibody
APS	ammoniumperoxidisulfate
A260	absorbance at 260 nm
b	base
bp	base pair
BCIP	5-Bromo-4-Chloro-3-indolylphosphate
BSA	bovine serum albumin
cAMP	adenosine 3',5'-cyclic monophosphate
CAR	cAMP receptor
CMF	conditioned medium factor
Ci	Curie
cpM	counts per minute
cDNA	complementary DNA
ddNTP	didesoxynucleotide, ddATP, ddCTP, ddGTP, ddTTP
dNTP	desoxyribonucleotide, dATP, dCTP, dGTP, dTTP
DIF	differentiation inducing factor
DTT	1,4-dithiotreitol
DEPC	diethylpyrocarbonate
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
DNase	desoxyribonuclase
EDTA	ethylendiamintetraacetic acid
G418	geneticin
GC	guanylyl cyclase
GDT	growth-differentiation transition
G-protein	heterotrimeric GTP binding protein
h	hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid
HRPO	horseradish peroxidase
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilo base pairs
kDa	kilo Dalton

lacZ	β-Galactosidase gene
mAb	monoclonal antibody
MCS	multiple cloning site, polylinker
MES	2-(N-Morpholino)-ethansulfonic acid
β-ME	beta-mercaptoethanol
min	minute
MOPS	γ-(morpholino)-propansulfonic acid
NP40	ethylenphenylpolyethylenglycol
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDE	phosphodiesterase
PDI	phosphodiesterase inhibitor
PEG	polyethylenglycol
PKA	cAMP dependent protein kinase
PLC	phospholipase C
PSF	pre-starvation factor
PIPES	1,4-piperazindiethansulfonic acid
PMSF	phenylmethylsulfonylfluoride
RNA	ribonucleic acid
RNase	ribonuclease
RNasin	RNase inhibitor
NTP	ribonucleotide, ATP, CTP, GTP, TTP
RT	room temperature
sarcosyl	N-lauroyl-sarcosine
SDS	sodium dodecyl sulphate
Strep.	streptomycin
TCA	tri-chloroacetic acid
TBE	Tris/borate electrophoresis buffer
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
Tris	tris-(hydroxymethyl)-aminomethane
Triton X-100	octylphenylpoly-(ethylenglycolether)
Tween 20	polyoxyethylen-sorbitan-monolaurate
Vol.	volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

I. ZUSAMMENFASSUNG

Unter Nahrungsmangel gehen *Dictyostelium discoideum* Zellen von der einzelligen amöboiden Lebensform in einen vielzelligen Organismus über, der sich zuletzt in zwei Zelltypen differenziert, nämlich Stielzellen und Sporen. Dieser Prozeß wird durch verschiedene Signale gesteuert, die die differentielle Genexpression regulieren. Unter den ersten Genen, die bei diesem Wachstums-Differenzierungs-Übergang (growth-differentiation-transition, GDT) aktiviert werden, ist die discoidin I Genfamilie, die deshalb auch als ein exzellenter Indikator für den Beginn der Entwicklung dient.

Mit Hilfe von Mutanten, die Defekte in bekannten Signaltransduktionskomponenten haben, und durch pharmakologische Analyse, konnten wir bereits ein Model für einen aktivierenden und einen inhibierenden Signalweg für die discoidinexpression aufstellen. Aktivierung erfolgt über einen unbekannten Rezeptor, ein heterotrimeres G-Protein, das die Gα2 Untereinheit enthält, CRAC (cytosolic regulator of adenylyl cyclase) und PKA (cAMP dependent protein kinase). Inaktivierung erfolgt über extrazelluläres cAMP, den cAMP Rezeptor cARI und Ca^{2+} Influx.

Um weitere Signaltransduktionskomponenten zu identifizieren, haben wir mit Hilfe der REMI-Mutagenese (restriction enzyme mediated integration) nach Klonen mit Discodin Fehlexpression (Unter- oder Überexpression) gesucht. Eine Mutation in einem neuen Gen, das *gdt1* benannt wurde, bewirkt discoidin-Überexpression und beschleunigte Entwicklung. In Western-Blots konnte gezeigt werden, daß das gdt1 Genprodukt ein 175 kDa membranassoziiertes Protein ist. Sequenzanalyse zeigte, daß der C-Terminus eine Kinasedomäne enthält, die zur Tyrosin-Rezeptorkinasefamilie gehört. Nach vorläufigen Daten ist gdt1 höchstwahrscheinlich an der Zell-Substrat-Adhäsion beteiligt. Dafür spricht die "RGD" Sequenz (Arg-Gly-Asp) in einer der extrazellulären Domänen. Wie durch Kombination der gdt1 Mutation mit den $G\alpha2^-$ und PKA^- Mutationen gezeigt werden konnte, reprimiert gdt1 die discoidin Genexpression, während die PKA Signalkaskade dem teilweise entgegenwirkt. Aus vorläufigen *in vitro* Experimenten ergibt sich, daß das gdt1 Genprodukt ein PKA Substrat sein könnte. Die gdt1 Rezeptorkinase ist ein negativer Regulator für discoidin und damit auch des GDT. Der gdt1 Signalweg verläuft parallel zu dem bereits zuvor identifizierten Gα2-CRAC-PKA Signalweg, wird jedoch auch durch PKA reguliert, und zwar höchstwahrscheinlich durch Phosphorylierung von gdt1. Eine Phosphorylierung durch PKA könnte das gdt1 Protein inaktivieren, was zu maximaler discoidin Transkription führen würde. Zerstörung des gdt1 Gens resultiert dementsprechend in deregulierter discoidin Überexpression und beschleunigter Entwicklung.

I. SUMMARY

When cells of *Dictyostelium discoideum* are deprived of nutrients, they undergo a transition from single cell amoebae to a multicellular organism which finally differentiates into two cell types: stalk-cells and spores. This process is organised by various signals which regulate the differential expression of genes. The discoidin I gene family is among the first genes activated at the growth-differentiation-transition (GDT) and thus serves as an excellent indicator for the beginning of development.

Using mutants with defects in known signal transduction components and pharmacological analysis, we have previously established a model for an activating and an inactivating pathway for discoidin expression: Activation occurs via an unknown surface receptor, a heterotrimeric G-protein containing the Gα2 subunit, CRAC (cytosolic regulator of adenylyl cyclase) and PKA (cAMP dependent protein kinase). Inactivation occurs via extracellular cAMP, the cAMP receptor cAR1 and Ca^{2+} influx.

By REMI mutagenesis (restriction enzyme mediated integration) we have screened for clones displaying mis-expression of discoidin (null or overexpression) to identify further signalling components. One mutant is disrupted in a new gene denominated *gdt1* resulting in overexpression of discoidin and accelerated development. Western-blots show that the gdt1 gene product is a 175 kDa membrane-associated protein. Sequence analysis indicates that the C-terminus contains a kinase domain which belongs to the Tyrosine Receptor Kinases family. Preliminary data shows that gdt1 is possibly involved in cell-substratum adhesion as suggested from the "RGD" (Arginine-Glycine-Asparagine) site at one of the extracellular domains. As demonstrated by combinations of the gdt1 mutation with $G\alpha2^-$ and PKA^- mutations, the gdt1 generates a repression of discoidin expression and is partially counteracted by the PKA signalling cascade. *In vitro* experiments suggest that the gdt1 gene product is a PKA substrate. The gdt1 receptor kinase is a negative regulator of discoidin and of GDT. The gdt1 signalling pathway is in parallel with the identified Gα2-CRAC-PKA pathway but is regulated by PKA, most likely, by PKA phosphorylation on gdt1. Phosphorylation by PKA may inactivate the gdt1 protein and allow the maximal level of discoidin transcription. Similarly, disruption of gdt1 results in deregulated discoidin overexpression and accelerated development.

II. INTRODUCTION

1. *Dictyostelium discoideum* as a model system to study development

For all eukaryotic systems, cellular differentiation proceeds through a series of stages that have to be carefully timed to result in the proper tissues and structures. In multicellular organisms, where organs are generated from different cell types, the processes must be temporally co-ordinated among diverse cells.

Usually a specific cell type cannot proceed to the next stage in differentiation until a signal is received from adjacent cells. *Dictyostelium discoideum*, which serves as a model system for the study of development, is an unique eukaryotic developmental system in which multicellularity arises from the aggregation of individual cells. The life cycle of *Dictyostelium* consists of distinct growth and developmental phases, and it is a simple matter to switch between the two modes. When nutrients are plentiful, *D. discoideum* cells grow and divide as individual amoebae. When cells are depleted of nutrients, growth ceases and development is initiated. Development is characterised by a stage in which around 10^5 single amoebae aggregate to form a multicellular organism and cells finally differentiate into two types: vacuolated stalk cells and durable spores (see Fig. 1).

In comparison to other eukaryotic organisms, *Dictyostelium* has some special biochemical and genetic characteristics. Such as:

→ Free-living *Dictyostelium* amoebae are easily grown and maintained (Sussman, 1987), thus clonal cells can be simply identified on a bacterial lawn. Some strains of *Dictyostelium discoideum* can also grow in axenic medium. This gives the possibility to carry out gene technical and cell biological work on cells growing in culture.

→ The differentiation of *Dictyostelium* is completely reversible until the late stages of the program. Growth stops when the nutrients in the environment are exhausted and there is no further replication of the chromosome until a fresh food source is found (Shaulsky *et al*., 1996). On re-introduction of nutrients, cells ″erase″ their developmental markers and resume growth (Soll and Finney, 1987). Thus, once developmental mutations are selected, they can be propagated and maintained, and the phenotypes can be expressed again when development is re-initiated.

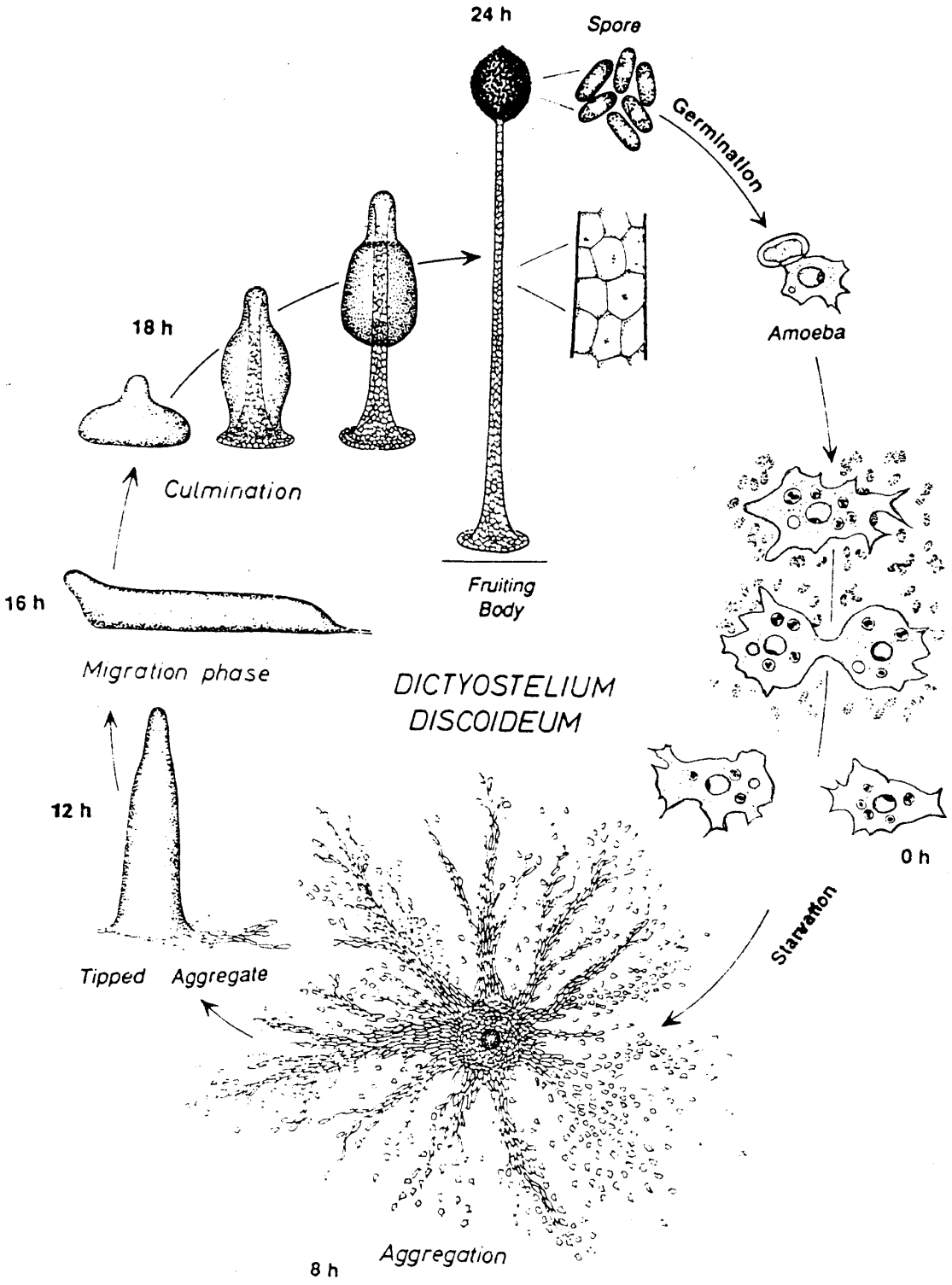

Figure 1. The life cycle of *Dictyostelium discoideum.* *Dictyostelium* is an unique eukaryotic developmental system in which multicellularity arises from the aggregation of individual cells. This process is initiated upon starvation, with vegetative growth and multicellular development being distinct phase of the life cycle. Because *Dictyostelium* generates single clones when fed on its natural food, bacteria, it is possible to screen for clonal *Dictyostelium* mutants with developmental phenotypes on bacterial plate.

→ *Dictyostelium* cells are haploid thus non-lethal recessive mutations can be directly scored. It is relatively easy to mutagenize cells, and selection of mutations is limited only by the screening or selection procedure (Loomis and Gilpin, 1987). However, *Dictyostelium* parasexual diploids can also be obtained by cell fusion. Recessive sensitivity markers were used to select against the haploid cells and allow survival of diploids only (Loomis, 1987). Thus, by use of tester strains marked with recessive resistance markers, mutations can be assigned to complementation and linkage groups (Welker, 1986). More recently, a true sexual mating system for *Dictyostelium* has been established for laboratory work (J. Williams, unpublished data).

Due to the above characteristics, *Dictyostelium discoideum* has long been serving as a useful system in which to study many of the complex processes of development (reviewed in (Loomis, 1975; Loomis, 1982), such as chemotaxis (Gerisch *et al*., 1987; Hall *et al*.,1989; Berlot *et al*.,1987), intercellular communication (Devreotes, 1989), cellular differentiation (Gomer *et al*., 1987; Coffman *et al*., 1993), and signal transduction (reviewed by Firtel, 1991; Devreotes, 1989). The movement and chemotactic response of *Dictyostelium* cells closely resemble those of amoeboid-like cells in higher eukaryotes such as leukocytes and macrophages (Zigmond, 1986), and the signalling properties of individual cells is more clearly understood in *Dictyostelium* than in other organisms.

2. Signal Transduction in *Dictyostelium*

The developmental process of *Dictyostelium* is controlled by cell-cell signalling, by which extracellular stimuli elicit intracellular responses. Various soluble factors are secreted by *Dictyostelium* and serve as signals controlling gene expression, developmental initiation, morphogenesis and cell differentiation (Devreotes, 1982). During exponential growth, most developmental gene products are present at low levels, except a group of V genes which are expressed only in vegetative stage and shut off upon differentiation (Singleton *et al*., 1991). A factor denominated PSF (‘prestarvation factor’) is continuously secreted into the extracellular medium by growing cells to measure the cell density (Clarke *et al*., 1988). On the other side, the bacteria upon which the cells feed inhibit the response to PSF, allowing the cells to monitor their own density in relation to that of their food supply (Clarke *et al*., 1992). Since both PSF and its receptor are not yet cloned, this pathway remains unclear. So far we know that at high PSF/bacteria ratios, which occur during late exponential growth, PSF induces the

expression of several genes whose products are needed for cell aggregation, such as the I-genes, discoidin I, and K5 (Grabel *et al*.,1978; Cardelli *et al*.,1985; Singleton *et al*., 1988). When the food supply has been depleted, PSF production declines, and a second density-sensing pathway is activated. Starving cells secrete conditioned medium factor (CMF), a glycoprotein (Gomer *et al*., 1991) essential for the development of differentiated cell types. CMF activates a so far unknown receptor, and generates an intracellular signalling which leads to cell-cell contacts. Using antisense mutagenesis it has been shown that cells lacking CMF cannot aggregate (Clarke and Gomer, 1995), suggesting that CMF regulates cAMP signal transduction (see below).

Cyclic AMP regulates further cellular differentiation in *Dictyostelium* leading to final morphogenesis. A highly organised network has evolved in *Dictyostelium* to result in intercellular signalling by pulses of cAMP coupled to a chemotactic response (Devreotes, 1982). Approximately 3-4 hours after removal of the food source, a small percentage of cells within a population begins emitting pulses of cAMP. Surrounding cells respond by moving chemotactically towards the signalling cells and by relaying the signal, which is accomplished by activation of adenylyl cyclase and release of cAMP into the surrounding medium. This in turn activates cells further away from the initiating cells and results in the movement of cells in a pulsatile cAMP gradient towards the aggregation center (Firtel *et al*., 1989; Devreotes, 1989). About 50 movement steps result in the formation of a multicellular structure (Gerisch *et al*., 1987; Schaap, 1986). The enzyme responsible for the synthesis of cAMP from ATP, adenylyl cyclase (ACA), rapidly accumulates during the first 8 hours of development and is activated when cAMP binds to a specific cell surface receptor, cARI (Klein *et al*., 1988). cARI is a serpentine seven-transmembrane protein coupled to heterotrimeric G proteins in a manner similar to the ß-adrenergic receptor of vertebrate nerve cells (Klein *et al*., 1988; Saxe and Kimmel, 1988). When cAMP binds to its extracellular domain, the cytoplasmic domain is activated and leads to the exchange of GTP for GDP bound to the Gα2 subunit (Firtel, 1991). The GTP form of Gα2 dissociates from the Gβγ trimeric complex and then activates adenylyl cyclase in conjunction with another protein, CRAC (Insall *et al*., 1994). CRAC carries a PH domain that may mediate its interaction with other proteins. However, the signalling via ACA is not yet fully understood. Within a minute of addition of cAMP to the cells, the activity of ACA can be increased 10 fold while this activity decreases with a half life of 2 minutes. This can result in a burst of cAMP synthesis, and most of the newly made cAMP is secreted for relaying the signal to adjacent cells (Mann *et al*., 1997). Cells lacking ACA or CRAC due to

the disruption of the *acaA* or *crac* genes, fail to aggregate or to show any sign of morphogenesis (Pitt *et al*., 1992). In addition to extracellular cAMP serving as a first messenger, intracellular cAMP functions as a second messenger in *Dictyostelium* like in other eukaryotes. It appears that all the essential responses to cAMP as an internal second messenger are mediated by the cAMP dependent protein kinase. In *Dictyostelium,* PKA plays multiple roles throughout development affecting chemotaxic aggregation, prespore and prestalk differentiation, terminal differentiation, as well as the growth-differentiation-transition (Simon *et al*., 1989; Mann *et al*., 1992; Harwood *et al*., 1992; Parent and Devreotes, 1996; Endl *et al*., 1996).

3. PKA activity appears to be the major effector of cAMP as a second messenger

A wide range of developmental processes in *Drosophila*, vertebrates and *Dicyostelium*, are mediated by PKA. In both *Drosophila* and zebra fish, PKA functions in signal transduction pathways initiated by the intercellular signalling protein *hedgehog* (Li *et al*., 1995; Jiang and Struhl, 1995). In *Drosophila* and mammals (Davis *et al*., 1995) PKA activity also plays a central role in the learning and memory system. The amino acid sequence of both C and R subunits are highly conserved among diverse organisms and the basic enzymatic properties are similar (Meinkoth *et al*., 1993). PKA is a pleiotropic protein kinase and regulates signalling pathways at multiples levels. In mammalian cells, ligand binding to receptors can result in fast activation of cytoplasmic PKA (Hagiwara *et al*., 1993) and in a slower translocation of PKA to the nucleus where it activates gene transcription by phosphorylating CREB (Mayo *et al.*, 1995) and NFκB (Verma, 1995). In *Dictyostelium*, there are multiple signal transduction pathways starting out from surface receptors, and most of them appear to use PKA as the central component. *Dictyostelium* PKA is held in an inactive form by association of a single catalytic subunit (PKA-C) with a single regulatory subunit (PKA-R) thus resulting in an holoenzyme RC. The binding of two molecules of cAMP to the R subunit results in the release of the C subunit. Dissociation of holoenzyme is complete when the intracellular concentration of cAMP raises above 100 nM. Though expression of *acaA*, *carA* (Mann and Firtel, 1991; Mann *et al*., 1997) and discoidin Iγ (Endl *et al*., 1996) have been shown to be regulated by PKA, no transcription factor has yet been determined to be a direct PKA substrate.

Both PKA-C and PKA-R are present at low levels in exponentially growing cells of *Dictyostelium*, but both accumulate about four-fold during the first 12 hours of development and remain at this level until culmination (Leichtling *et al*., 1984; Part *et al*., 1985). The mRNA levels of both *pka*C and *pka*R increase about five-fold during the first four hours of development and then remain constant throughout development (Burki *et al*., 1991; Simon *et al*., 1989). The accumulation of both C and R subunits are co-ordinated, and there is no evidence for differential accumulation of either subunit during aggregation or in the two major cell types at the slug stage (Vaughan and Rutherford, 1987). Overexpression of the C subunit results in a rapid development: cells forming spores in as little as 16 hours, and expression of some early developmental genes, like discoidin, are accelerated (Anjard *et al*., 1992). The R subunit was mutated in both cAMP binding sites, resulting in a stable dominant inhibitor of PKA (Rm). Strains overexpressing Rm during vegetative growth fail completely to develop (Simon *et al*., 1989). The same phenotype is also expressed by the PKA C^- strain, which carries a null mutation of the C subunit (Mann and Firtel, 1991). PKA plays a core role on the whole developmental process of *Dictyostelium*. However, there is as yet no evidence on its function neither in exponential growth nor at the growth-differentiation-transition. Both Rm and PKA C^- strains grow well in axenic medium, but the PKA C^- strain grows slower than wild type cells on bacteria (Kessin and Anjard, personal communication). This suggests that PKA is not essential for growth but is involved in the signalling pathways related to the food source. The regulation of the very early developmental gene discoidin by PKA strongly supports this.

4. The discoidin I gene family is an indicator for the onset of differentiation in *Dictyostelium*

As *Dictyostelium discoideum* amoebae differentiate from the non-cohesive to the cohesive state, they synthesise two galactose-binding lectins, discoidin I and II. These two proteins have common regions (49% identity in the amino acid sequence): a carbohydrate-binding site (Berger and Armant, 1982) and an Arg-Gly-Asp (RGD) sequence which has been found in vitronectins and fibronectins in mammalian cells (Pierschbacher and Ruoslahti, 1984). discoidin I is expressed at the growth-differentiation-transition (GDT), and concentrated in the slime coat and around stalk cells (Devine *et al*., 1982; Cooper and Barondes, 1984). discoidin II is expressed at the onset of aggregation (8 h of development), and prominent in and around prespore cells (Cooper and Barondes, 1984; Fukuzawa and Ochiai, 1996). There are three members in the discoidin I gene family, which begin to coordinately accumulated approximately three generations before the onset of development (Devine *et al*., 1982). Thus

the discoidin I gene family sense as a marker to study the regulation of GDT. Monoclonal antibodies directed against the discoidin I proteins have been generated (Fukuzawa and Ochiai, 1988; Wetterauer *et al*., 1993).

The functions of the discoidin I protein are not clear. For a long time it was believed that discoidin I is implicated in cell to cell cohesion because it can bind to galactose and has a ″RGD″ site. RGD is the minimal structure recognised by mammalian cells in fibronectins and vitronectin, and constitutes the cell attachment sites of these proteins (Pierschbacher and Ruoslahti, 1984; Suzuki *et al*., 1985). Peptides containing this ″RGD″ signal specifically prevent gastrulation in *Drosophila* embryos (Naidet *et al*., 1987), suggesting that the RGD-sequence may also be used by invertebrates to mediate cell-attachment. In *Dictyostelium,* it has been reported that synthetic peptides containing RGD block cell attachment and spreading on a plastic surface and formation of fruiting bodies (Springer *et al*., 1984). However, immunofluorescence analysis demonstrated that discoidin is distributed throughout the cytoplasm excluding vesicles and nuclei, and there is no indication for either extracellular or membrane localization (Alexander *et al*., 1992). The discoidin proteins are essential for the establishment of the elongated cell morphology, cytoskeletal organisation and the side-to-side alignment of cells during aggregation (Alexander *et al*., 1992). However, the elongated morphology is not a required for rapid and persistent single cell translocation.

At the growth-differentiation-transition (GDT), the discoidin I gene family is among the first activated genes and thus considered as an indicator for the onset of differentiation in *Dictyostelium*. discoidin I serves as a facultative marker of GDT: expression is neither required for development nor does discoidin expression lead to obligatory development. This is demonstrated by disc$^-$ mutants which undergo relatively normal development (Crowley *et al*., 1985; Alexander *et al*., 1986) and by the continuous expression of discoidin in axenic growth medium (Blusch *et al*., 1995). The transcription level of discoidin I is very low when cells feed on sufficient bacteria. Several generations before the on-set of starvation, the PSF factor, which is continuously secreted by the *Dictyostelium* cells, accumulates in the extracellular medium. Above a threshold level of PSF, the expression of discoidin is induced (Rahti and Clarke, 1992). Deviation of this induction pattern can be monitored by measuring discoidin expression at different cell densities (Wetterauer *et al*., 1995). When the food source is exhausted and cells stop growing, a strong induction of discoidin occurs. However, the other factor CMF, which is secreted when cells starve and which activates cell differentiation,

is not responsible for the induction of discoidin. As will be presented in this thesis, a CMF knock-out strain shows normal discoidin expression. Thus another signalling pathway regulates accumulation of discoidin. Later, after 6 hours of development, the high level expression of discoidin is turned off by cAMP, which is synthesised and secreted by cells and is involved in multiple signalling pathways throughout development. A model for discoidin I gene regulation (see Fig. 2) has been presented previously and some components required for regulated expression of the discoidin I genes have been identified (Blusch *et al*., 1992; 1995; Endl *et al*., 1996). An intracellular cAMP signal is generated through a pathway including the G protein α2 subunit, CRAC, an unknown adenylyl cyclase and the activation of cAMP dependent protein kinase (PKA). PKA then stimulates transcription of discoidin I by an unknown phosphorylation linkage (Fig. 2). Mutants with aberrant discoidin expression have been generated to obtain insights into the regulation of GDT, among them are the VI88 strain (Wetterauer *et al*., 1993) and the drsA strain (Alexander *et al*; 1983) which show a discoidin overexpression phenotype. However, these mutants were generated by chemical mutagenesis thus the molecular basis is not yet known. So far, components involved in the signalling network towards regulation of discoidin and the interactions between different pathways are still incompletely understood.

By using 5' deletions in the promoter analysis vector PAV-CAT (May *et al*., 1989) and different artificial gene fusions, several sequence elements have been identified to be the ultimate targets for extracellular signals, and to relay signalling to the transcription of the discoidin I gene. The promoter element dIE (discoidin induction element) which displays some characteristics of an enhancer, responds to both the prestarvation factor PSF (Vauti *et al*., 1990) and the folate repression signalling (Blusch *et al*., 1992). Another element, dNCE (discoidin negative cAMP element), located downstream of the dIE is necessary for down regulation by the cAMP signalling (Vauti *et al*., 1990). Further down stream in the discoidin promoter, there is another element dAE (discoidin axenic element) which is required to maintain the basic expression of discoidin during axenic growth (Vauti *et al*., 1990). Identification of these promoter elements provides the basis for tracing back the transduction chain from the level of gene expression to the events at the membrane.

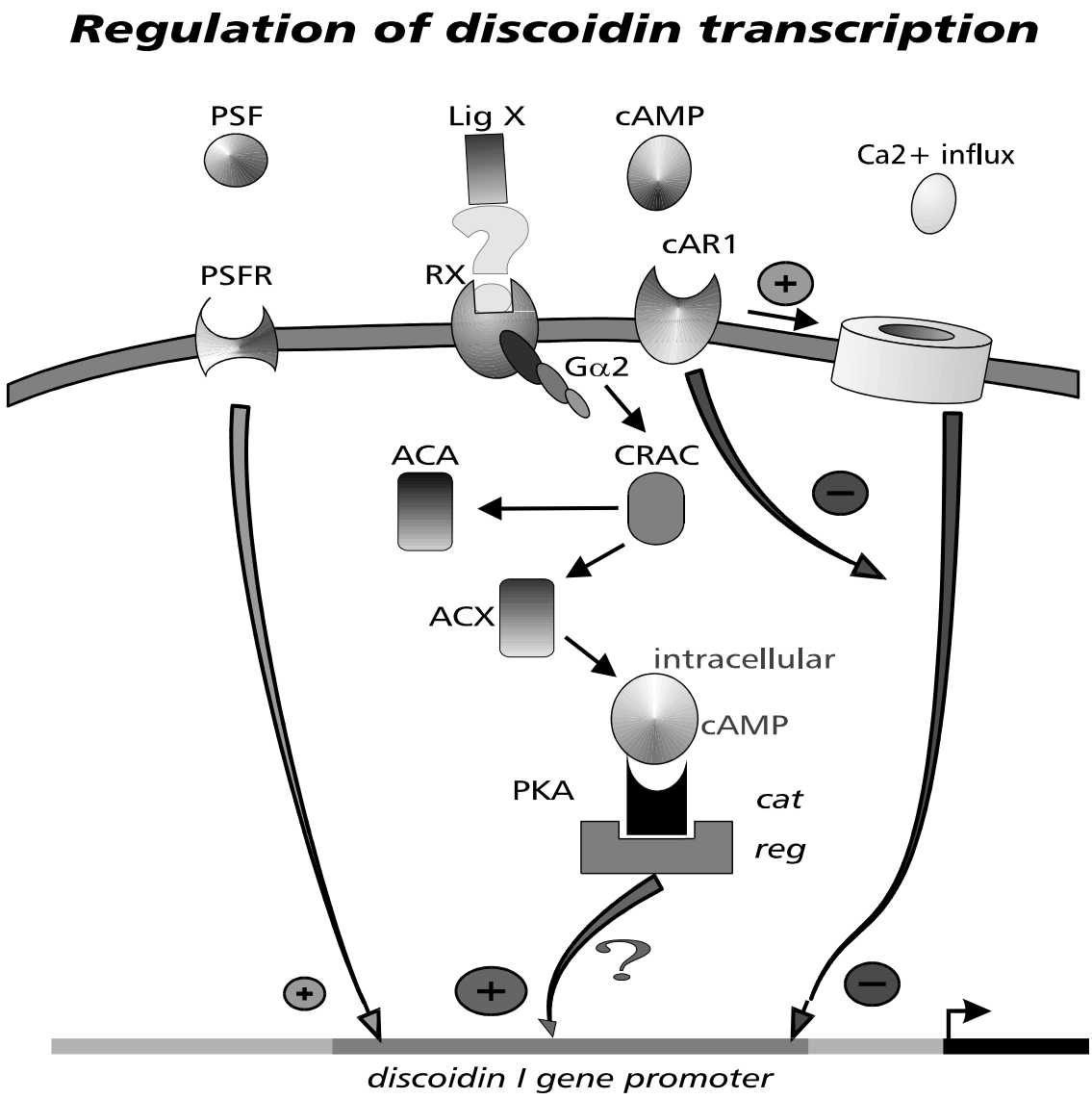

Figure 2. **Initial model for the regulation on discoidin transcription** [from Endl and Nellen, 1996]. Four different regulatory pathways were proposed: PSF and an unknown molecule (ligand X) induce discoidin expression by intracellular signalling through their receptors (PSFR and RX). The pathway by RX involves Gα2, and CRAC which is known to activate ACA. However, ACA was found not to be required for discoidin induction. Therefore another adenylyl cyclase (ACX) was proposed. ACX generates cAMP which activates PKA. The final activation of discoidin transcription via PKA is unknown. Extracellular cAMP and Ca^{2+} influx generate repressing regulations and counteract the induction. Membrane associated proteins like ACA and CRAC are shown inside the cell only for graphical reasons.

5. Using the REMI strategy and discoidin I as a marker to knock out developmental genes at the growth-differentiation-transition of *Dictyostelium*

A new technique for fishing genes by tagged insertion mutagenesis has been established in *Dictyostelium* (Kuspa and Loomis, 1992). Introduction of a restriction enzyme along with a linearized plasmid results in integration of the plasmid at corresponding genomic restriction sites. This method, called REMI (restriction enzyme mediated integration), generates apparently random insertions into genomic DNA. Some integrations cause gene disruptions and thus mutations. After screening for desired phenotypes, the disrupted genes can be isolated together with the inserted plasmid and analyzed. In the next step, to prove that the disruption of the gene by the inserted plasmid is responsible for the observed phenotype, the isolated plasmid is reused to disrupt the gene in wild type cells by homologous recombination. Since development and vegetative growth are distinct phases, it is possible to obtain mutants defective in important signal transduction pathways without impairing the viability of the cells. This REMI system was used to isolate mutants with defects in the expression of discoidin genes.

As described in Fig. 3, an ura$^-$ auxotroph strain of *Dictyostelium* (DHI) was used as a parent for generating the REMI mutants. The DIV-2 vector, which contains the pGem-3 vector and *pyr5-6* gene (coding for UMP synthase) is linearized with *Bam*HI and electroporated into the competent DHI cells together with *Bam*HI. The enzyme cuts occasionally the genomic DNA and the vector is randomly integrated into the genome at the cut site(s). Transformants carrying the *pyr5-6* gene are selected in minimum medium without uracil for about 15 days, then cloned on a bacterial lawn. The REMI mutants with mis-expression of the discoidin I genes were obtained by colony-blot screening with anti-discoidin monoclonal antibodies. The identified mutants were analyzed by Southern-blots probed with the pGEM-3 vector to confirm the altered genomic structure caused by the integration. The disrupted gene was then isolated by a proper restriction digestion which recovers the pGEM-3 vector part (containing the origin of replication and the Amp resistance) of the DIV-2 vector and the flanking genomic DNA at the integration site. This step of recovering the flanking genomic fragments is called REMI rescue. The isolated fragments were then analyzed and used for reconstructing the phenotype.

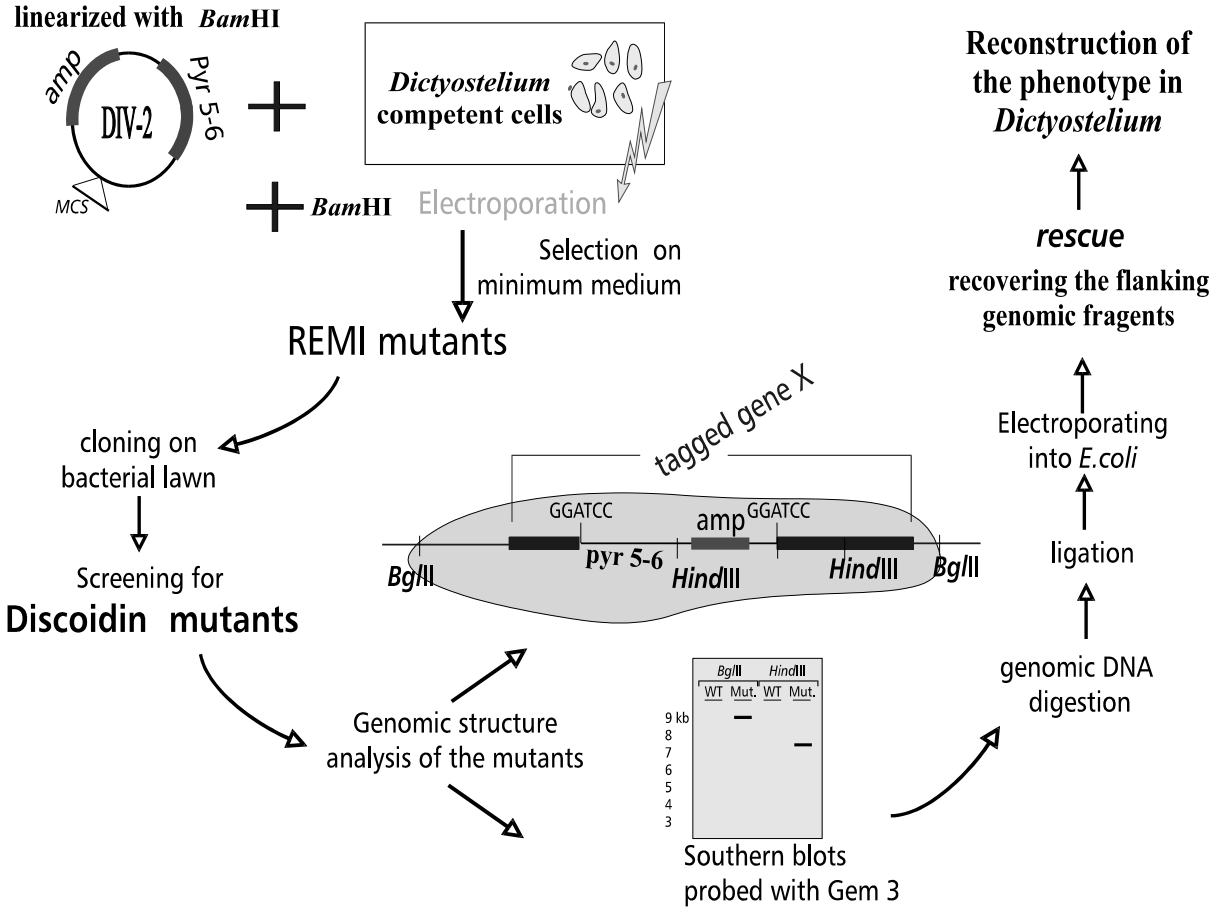

Figure 3. Diagram of REMI strategy for fishing genes involved in the regulation of discoidin. The linearized DIV-2 vector together with *Bam*HI enzyme is electroporated into the competent DHI (ura$^-$ auxotroph strain) cells. The enzyme cuts occasionally inside the genome and the vector is randomly integrated. Transformants are selected in minimum medium then cloned on a bacterial lawn. The mutants with mis-expression of discoidin I are screened by colony-blots with an anti-discoidin monoclonal antibody. The flanking genomic DNA at the integration site of the identified mutant is isolated by a proper restriction digestion together with the pGEM-3 vector. This step of recovering the flanking genomic fragments is called REMI rescue and the isolated fragments are then analyzed and used for reconstructing the phenotype.

By REMI, both discoidin^{null} and discoidin^{over} mutants have been isolated, among them formerly determined components like CRAC (Riemann, unpublished data). This work will allow to dissect the signal transduction pathways which trigger the transition from cell proliferation to cell differentiation.

6. Protein phosphorylation and protein kinases

Since the *gdt1* gene encodes a putative protein kinase, this chapter will give a brief introduction on the general concept of protein phosphorylation and protein kinase.

Of all the post-translational modifications, protein phosphorylation plays the most dominant role in almost all events in cellular regulation, including proliferation, differentiation, signal transduction, metabolism and cell death. Phosphorylation of a target protein is regulated by the balance of activity of protein kinases and protein phosphatases. The activity of protein kinase and phosphatase is modulated both temporally (different time scales) and spatially (different cellular and subcellular locations). Some protein kinases have multiple substrates and can act at multiple sites. These properties provide an extraordinarily sensitive regulation for physiological functions, while the activity of a protein can be enhanced or diminished in a quick and reversible manner. In a typical mammalian cell, an estimated one-third of all proteins are phosphorylated, and approximately 2-5% of the genes encode protein kinase and phosphotases (Hubbard and Cohen, 1993). To date, more than 400 protein kinases and more than 100 protein phosphatases have been identified (Taylor *et al*., 1995; Hunter, 1997).

The signal transduction cascade involves a large number of protein kinases, by which the primary signal is amplified and the complex cellular regulatory pathways are generated. However, the cellular response of phosphorylation on a signalling component is quite diverse. Some enzymes require phosphorylation for activity (e.g., phosphorylase b), while the others are inactivated by phosphorylation (e.g., glycogen synthases and pyruvate kinase) (De Meyts *et al*., 1995; Denton and Tavare, 1995). Phosphorylation provides regulation in a variety of ways, including allosteric activation or inhibition, the formation of protein-protein interactions, and protein translocation from one compartment of the cell to another (Jans, 1995). Both extracellular ligands, such as polypeptide growth factors, and key intracellular signalling elements such as the concentration of cAMP or calcium regulate the activities of kinase and phosphatase, which themselves are subject to modification by phosphorylation (e.g. PKA) .

The inherent complexity of signal transduction pathways is intensively analyzed in the activation of the mitogen-activated protein kinase (MAPK) cascade (Seger and Krebs, 1995). The MAPK pathway involves a series of serine/threonine protein kinases or serine/tyrosine protein kinases, where phosphorylation of the next kinase in the series results in its activation.

Signalling through this cascade results in approximately 10,000 fold amplification of the initial signal. During the course of this signalling, other signalling pathways participate and either stimulate (e.g., PKC signalling) or inhibit (e.g., PKA signalling and protein phosphatase activity) the MAPK pathway. An activated MAPK enzyme in turn stimulates a variety of cytosolic enzymes (e.g., glucose metabolism) and also transfers to the nucleus, where it further phosphorylates / activates a series of transcription factors (Graves *et al*., 1995).

Many cytokines initiate cellular responses through their interaction with members of the cytokine receptor superfamily which contain no catalytic domains in their cytoplasmic domains. Irrespective, ligand binding induces tyrosine phosphorylation, which requires a membrane proximal region of the cytoplasmic domain. The Janus kinase (JAK) family, which belongs to the protein tyrosine kinases, associates with the membrane proximal region and are rapidly phosphorylated following ligand binding and their kinase activity is activated (Ihle, 1994; Xia *et al*., 1996). Moreover, different cytokines activate the same JAK kinase and the same cytokine ligand can activate more than one member of the JAK superfamily (Foxwell *et al*.,1995; Gaffen *et al*., 1996). The Jak-STAT pathway is a newly discovered intracellular signal transduction pathway that is used by a growing number of extracellular signalling proteins (ESPs) for transcriptional activation of target genes. The activated receptor-JAK kinase complexes recruit members of the STAT family and activate them by phosphorylation. As a consequence, the phosphorylated STAT proteins dimerize, translocate into the nucleus, bind response elements in the promoter of target genes and stimulate the transcription of these genes (reviewed by Heim, 1996).

Protein kinases can be classified according to their functional properties. One categorisation is based on the specific amino acid residue that serves as the phospho-acceptor. This includes the serine-threonine protein kinases, which transfer the phosphate group onto serine and /or threonine residues; the protein tyrosine kinases (PTKs), which transfer the phosphate group onto tyrosine residues; and the dual specificity protein kinase, which transfers the phosphate group onto serine, threonine and tyrosine residues (Taylor *et al*., 1995). A second classification scheme categorises kinases based on their requirement for second messengers (e.g. cAMP), but the boundaries between groups are less definite. Table 1. provides some examples (by Promega) on various classes of protein kinases.

Table 1. Classification of protein kinases based on second messenger requirement.

Messenger-Independent Protein Kinase (Cofactor)	
Enzyme class	**Examples**
Receptor Serine/Threonine PK	transforming growth factor β (TGFβ) receptor and activin receptor, etc.
soluble Serine/Threonine PK	Casein kinase 1 and 2, MAP kinase (ERKs, p38, JNK), MEKs, MEKKs, etc.
Receptor Tyrosine Kinase (RTKs)	Insulin receptor (IR), epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), nerve growth factor receptor (NGFR or TRK), fibroblast growth factor receptor (FGFR), etc.
Messenger-Dependent Protein Kinase	
Enzyme	**Second Messenger**
PKA	cAMP
JAK	cytokines
cyclic GMP-dependent PK (PKG)	cGMP
Calcium and phospholipid-dependent PK	Ca^{2+}, diacylglycerol (DAG), and phospholipids
Calmodulin-dependent PK (CaMK)	Ca^{2+}, Camodulin (CaM)
DNA-dependent PK (DNA-PK)	dsDNA
dsRNA-dependent PK (RNA-PK)	dsRNA
phosphorylase kinase (Phos K)	Ca^{2+}, Camodulin (CaM)
Myosin light chain PK (MLCK)	Ca^{2+}, Camodulin (CaM)
Cyclin-dependent PKs (CDK)	Cyclins A, B, D, E