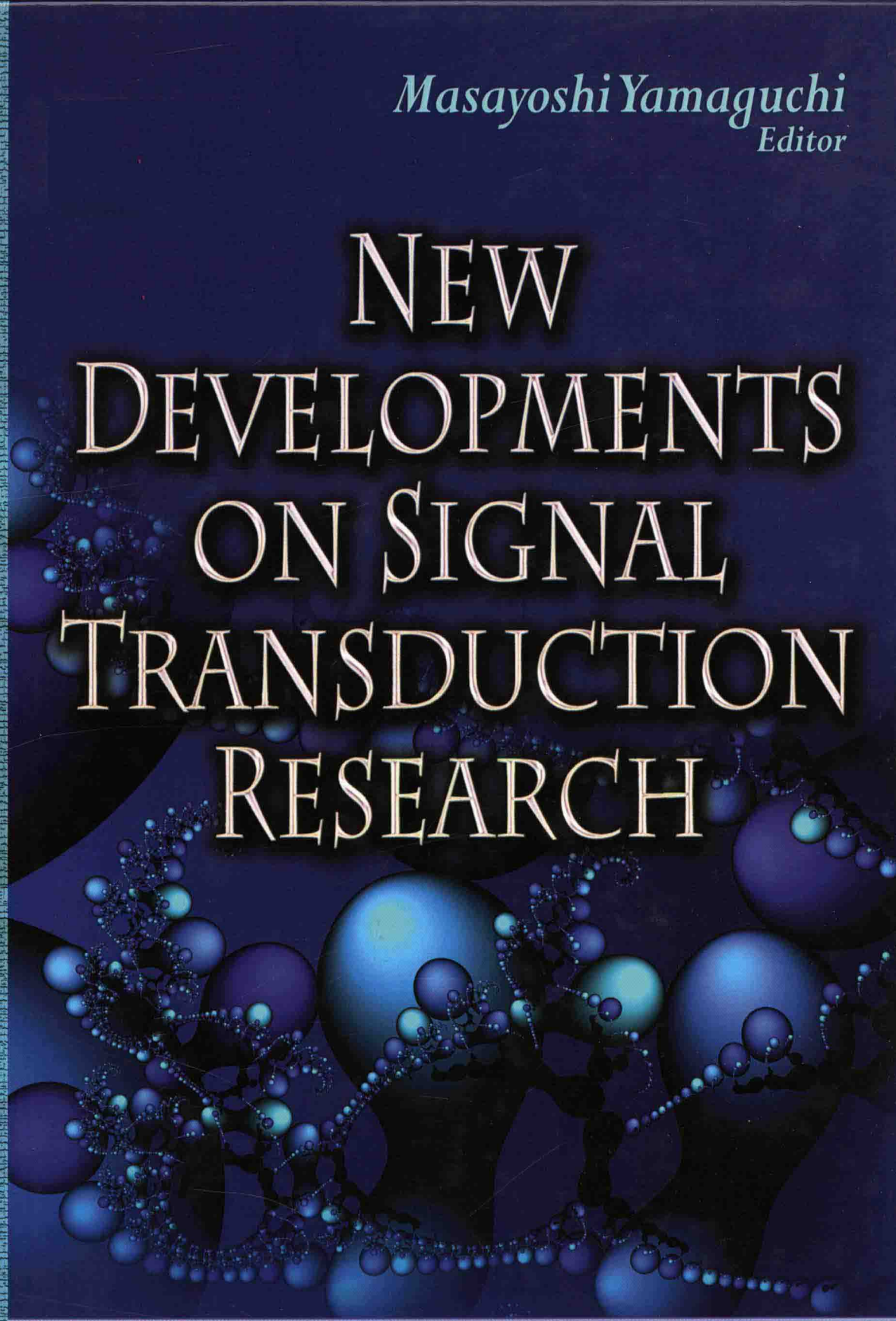
Masayoshi Yamaguchi
Editor

New Developments on Signal Transduction Research



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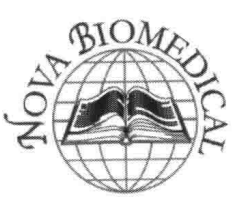
MASAYOSHI YAMAGUCHI
EDITOR



New York

PREFACE

Signal transduction plays a pivotal role in cell regulation. Hormones, cytokines and neurotransmitters bind to their specific receptors in the plasma membranes of cells. The receptors of many hormones are coupled to G-protein coupled signaling pathways. Insulin and growth factors are coupled to receptor-linked kinase. The second messenger is generated in cells once the first messenger binds to the receptors of plasma membranes. Cyclic adenosine monophosphate (cyclic AMP), inositol 1,3,5-triphosphate (IP3) and calcium ion (Ca^{2+}) have been demonstrated to play a role as a second messenger in cells. The pivotal role of Ca^{2+} in cellular regulation was established with the finding of calmodulin and protein kinase C that modulates the effect of Ca^{2+} in the regulation of cellular functions. Many receptor classes and their downstream kinases activate Erk, Akt, Stat, Smad, NF-κB and other cascades to regulate gene expression and cellular function. This book provides recent research in the study of the new developments in signaling transduction in cells.

Chapter 1 - Calcium signal transduction is central to the growth and survival of all cells. As an intracellular messenger, calcium primarily works by binding to and regulating a diversity of calcium-binding proteins, of which calmodulin (CaM) is the most common. The central role of the calcium-sensor CaM has been well chronicled in a vast diversity of cellular functions in animals, plants and eukaryotic microbes. In response to increased levels of calcium, apo-CaM binds up to four Ca^{2+} undergoing a major conformational change as Ca^{2+}-CaM. Both apo-CaM and Ca^{2+}-CaM each bind to a specific complement of CaM-binding proteins (CaMBPs). While the roles of intracellular CaM and its CaMBPs have been well documented, the existence as well as the functions of these as extracellular proteins has been both controversial and enigmatic. This review summarizes the roles of extracellular CaM (extCaM) and extracellular CaMBPs (extCaMBPs) in animals, plants and eukaryotic microbes. The authors work with the amoebozoan *Dictyostelium*, which is the primary focus of this chapter, has shown that extCaM serves several functions in this organism. It regulates growth and chemotaxis. It also binds to and inhibits the proteolysis of at least one extCaMBP, the cysteine-rich matricellular protein CyrA. The presence of extracellular CaM with proven targets and functions in a eukaryotic microbe suggests that extracellular CaM is as ubiquitous and important evolutionarily as cytoplasmic CaM.

Chapter 2 - The loss of neurons in neurodegenerative disease leads to devastating loss of function. Because of their ability to give rise to new neurons, neural progenitors offer hope for functional restoration of neurons in neurodegenerative disease, either through pharmacologic intervention that induces adult neural stem cells to differentiate in situ, or by

transplanting neurons differentiated in vitro. In order to advance these efforts, multiple groups are working to define the complex signaling pathways that govern neuronal differentiation. These pathways are activated by self-renewal and differentiation cues, typically in the form of growth factors or morphogens that bind to cell-surface receptors. Expression of growth factors and their receptors is dynamically regulated in the nervous system, establishing tightly controlled spatial and temporal gradients of activity. In the past decade, kinase-coupled receptors such as TGFβ, Lif, and FGF receptors have been clearly established as key regulators of stem cell multipotency and differentiation. These receptor classes and their downstream kinases activate Erk, Akt, Stat, and Smad cascades to regulate gene expression and cellular function. More recent studies highlight the overlooked importance of signaling through G protein coupled receptors (GPCRs), whose downstream effects overlap significantly with kinase-coupled receptors. Specifically, GPCRs have also been shown to regulate Smad, Stat, Akt and Erk pathways. The GPCR family is large and diverse, and specific GPCRs and their ligands have been shown to regulate stem cells from various sources, including neuronal stem cells. This chapter will highlight convergent kinase-coupled and G protein-coupled signaling pathways, and highlight examples of co-regulation of neural stem cell differentiation and multipotency by both receptor types.

Chapter 3 - For most moths, the ability of conspecific males to locate receptive females is governed by the detection of a blend of semiochemicals known as sex pheromones. Sex pheromone components are *de novo* synthesized in the female pheromone gland in response to pheromone biosynthesis activating neuropeptide (PBAN). The pheromonotropic effects of PBAN are mediated by a G protein-coupled receptor predominantly expressed in the pheromone gland, termed the PBAN receptor (PBANR). PBANR activation triggers the mobilization of extracellular Ca^{2+}, which activates a signal transduction cascade that activates the enzymatic processes involved in sex pheromone production. PBANR was initially identified in *Helicoverpa zea* and *Bombyx mori* based on sequence similarities with the mammalian neuromedin U family of receptors. Since then PBANRs have been identified in a number of other moth species. Functionally relevant structural variations suggested the presence of two PBANR isoforms, one expressed in moths using non-cAMP dependent biosynthetic pathways and the other in moths using cAMP dependent pathways. Recent studies, however, have shown that alternative splicing of the PBANR gene generates multiple variants, including the two initial isoforms that are concomitantly expressed in moth pheromone glands. Given the importance of sex pheromones to moth mating behaviors, PBANR has been suggested as a potential candidate for targeted disruption. This chapter provides a brief overview of PBANR identification and the role molecular dissection of the PBANR sequence has had in elucidating critical structure-function relationships. Recent developments in generating PBANR agonists/antagonists and the potential for RNAi interference-based disruption of PBANR functionality are also discussed.

Chapter 4 - Cyclin dependent kinase-5 (cdk5) belongs to the family of cyclin dependent kinases (cdks) based on its sequence homology, varying between 60-70 percent, with the other cdks. However, its activity is primarily restricted to neuronal cells. It is activated by neuron-specific molecules p35 and p39 with molecular weights of 35kD and 39kD, respectively, which have similar cyclin structures. Cdk5 has been shown to be a multifunctional protein kinase that regulates a large number of neuronal processes including synaptic activity, neural system development, and memory and learning. Cdk5 is not only necessary for nervous system development but is also essential for survival. The knockout of

cdk5 is embryonically lethal. It has been shown that cdk5 not only phosphorylates its substrates such as NF, tau, and other cytoskeletal molecules, but also regulates the activity of kinases and phosphatases by kinase-phosphatase cross-talk. In this review, the cross-talk of cdk5 with different kinases and phosphatases and their role in the nervous system function and development is described.

Chapter 5 - A wide variety of environmental chemicals and drugs can interfere with normal embryonic development, leading to malformations and other anomalies. While the underlying cellular and molecular mechanisms are not always clear, there have been numerous studies implicating disruptions in signal transduction pathways in the induction of abnormalities by embryotoxic compounds. The types of intracellular signalling pathways influenced by teratogens are quite diverse and, in many cases, the pathway is activated by treatment. The Mitogen-Activated Protein Kinase family (MAPK) includes members that influence both cell proliferation and cell death. Stress-induced members of this family (c-Jun N-terminal kinase, (JNK) and p38) have been found to be induced by exposure of vertebrate embryos to toxic metals, cyclophosphamide, hydroxyurea, perfluorooctane sulfonate (PFOS) and staurosporine. The activation of these stress proteins is often linked to reactive oxygen species and oxidative stress, and typically culminates in apoptosis. Activation of Ras, which typically leads to cell growth and differentiation, is involved in phenytoin embryopathy. Rather than being activated, other pathways are inhibited. The Protein Kinase B/Akt pathway, which is linked to cell survival, has been found to be down-regulated after limb cells are exposed to the well-known teratogen, thalidomide. Developmentally important signalling pathways, such as Sonic Hedgehog (Shh), are disrupted by exposure to ethanol and retinoic acid. The primary consequences of the induction of many of these pathways include alterations in cell proliferation, differentiation, apoptosis and cell migration. These changes, in turn, disrupt the normal processes of embryonic development, leading to malformations or embryonic death.

This chapter will discuss the major disturbances in cell signalling by treatments that interfere with embryogenesis, with the aim of determining whether commonalities exist among the toxins, outcomes and pathways affected that would help to explain the mechanisms of toxin-induced abnormal development.

Chapter 6 - Regucalcin was discovered in 1978 as a novel calcium-binding protein that does not contain EF-hand motif of calcium-binding domain, which differs from calmodulin and other calcium-related proteins. The name of regucalcin was proposed for this calcium-binding protein, which regulates various Ca^{2+}- or Ca^{2+}/calmodulin-dependent enzyme activations. Regucalcin and its gene (RGN) are identified in over 15 species consisting of regucalcin family. The regucalcin gene is localized on the chromosome X, and the organization of the regucalcin gene consists of seven exons and six introns. AP-1, NF1-A1, and RGPR-p117, which is a transcription factor, bind to the promoter region of the regucalcin gene and enhance transcription activity of the regucalcin gene expression that is mediated through calcium and other signalings. Regucalcin mRNA expression and its protein content are pronounced in the liver, kidney cortex, and other tissues. Regucalcin has been demonstrated to play a multifunctional role in cell regulation: regulation of intracellular Ca^{2+} homeostasis, inhibition of Ca^{2+}-dependent and independent enzyme activations including protein kinases and protein phosphatases, suppression of nuclear signaling-related deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis, and depression of apoptotic cell death and cell proliferation induced by various signaling factors in many cell

types in vitro. Morever, regucalcin has been shown to have a suppressive effect on protein synthesis and an activating effect on protein degradation. Regucalcin suppresses the over-proliferation in regenerating liver cells with partial hepatoectomy and the development of carcinogenesis in liver tissues in vivo. Regucalcin is proposed to play a pivotal role in maintaining of cell homeostasis and function. The revelation of these effects is related to role of regucalcin as a suppressor in various cell-signaling systems. Regucalcin is the fisrt time finding as a suppressor protein in cell signaling. This chapter has been written to outline the recent advances that have been made concerning the role of regucalcin as a suppressor protein in cell signaling in liver, kidney, and other tissues.

Chapter 7 - By use of Paraquat (PQ) treatment during germination of pea seeds the authors obtained a high yielding mutants in the preceding works. The germinating seeds of *Oryza sativa* cv Koshihikari were treated in the presence of 4 μM of PQ, and the authors isolated PQ resistant mutants, R4-2-4 and R4-5-8 with high yielding. The two mutant lines, R4-2-4 and R4-5-8 showed significantly increased tiller numbers, ear numbers and ear weights and the leaves were deeper in green color than those of original wild type line. The mutant R4-2-4 and R4-5-8 lines showed resistant phenotype to PQ at the concentrations of 1-4 μM exhibiting good growth ability with green color of cotyledon. However, at the same concentration of PQ the seeds of original wild type showed germination with white cotyledon with no further growth ability. The leaves from both mutants were isolated and the crude extracts were prepared and these fractions were phosphorylated followed by the SDS-PAGE fractionation.

The increase in the phosphorylation bands in low molecular weights corresponding to the precursor of nucleoside diphospate kinase (NDPK) were observed in both mutant lines, and also increase in the phosphorylation bands in the higher molecular weights corresponding to high molecular weight histidine kinases could be detected in both of the mutant lines.

However, in the original wild type increased amounts of putative degradation product of phosphorylated precursor of NDPK could be detected. By the further fractionation of the soluble, chloroplast and mitochondrial fractions, the increase in the phosphorylation of protein bands corresponding to putative precursor of NDPK and higher molecular weight histidine kinases in the fractions of chloroplast could be observed. The nucleotide sequence analyses of the precursor of NDPK1, 2 and 3 showed no difference in the nucleotide sequence with those of original wild type line and mutant lines, R4-2-4 and R4-5-8. However, the authors detected that 78 amino acid sequence spanning in the transit peptide of Nipponbare NDPK2 was deleted in the Koshihikari transit peptide of NDPK2. The yield tests of original wild type and a mutant R4-2-4 with Kinuhikari as a reference line performed in Kanagawa Agricultural Technology Center demonstrated higher yielding of 1.11-, 1.12- and 1.35-fold than those of original line.

The authors estimate that higher molecular weight histidine kinase and the putative precursor of NDPKs in cytosol and on chloroplast may have capacity to function in the process of detoxification of reactive oxygen species including singlet oxygen ($^1O_2$).

CONTENTS

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Chapter 1

SIGNAL TRANSDUCTION MEDIATED VIA EXTRACELLULAR CALMODULIN AND EXTRACELLULAR CALMODULIN BINDING PROTEINS

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ABSTRACT

Calcium signal transduction is central to the growth and survival of all cells. As an intracellular messenger, calcium primarily works by binding to and regulating a diversity of calcium-binding proteins, of which calmodulin (CaM) is the most common. The central role of the calcium-sensor CaM has been well chronicled in a vast diversity of cellular functions in animals, plants and eukaryotic microbes. In response to increased levels of calcium, apo-CaM binds up to four Ca^{2+} undergoing a major conformational change as Ca^{2+}-CaM. Both apo-CaM and Ca^{2+}-CaM each bind to a specific complement of CaM-binding proteins (CaMBPs). While the roles of intracellular CaM and its CaMBPs have been well documented, the existence as well as the functions of these as extracellular proteins has been both controversial and enigmatic. This review summarizes the roles of extracellular CaM (extCaM) and extracellular CaMBPs (extCaMBPs) in animals, plants and eukaryotic microbes. Our work with the amoebozoan *Dictyostelium*, which is the primary focus of this chapter, has shown that extCaM serves several functions in this organism. It regulates growth and chemotaxis. It also binds to and inhibits the proteolysis of at least one extCaMBP, the cysteine-rich matricellular protein CyrA. The presence of extracellular CaM with proven targets and functions in a eukaryotic microbe suggests that extracellular CaM is as ubiquitous and important evolutionarily as cytoplasmic CaM.

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Keywords: extracellular calmodulin, calmodulin binding proteins, *Dictyostelium*, social amoebozoan, chemotaxis, matricellular

INTRODUCTION

Calmodulin (CaM) is a ubiquitous calcium-binding protein found in essentially all eukaryotes studied to date. Calcium-free or apo-CaM undergoes a dramatic conformational change in response to binding between one and four calcium ions as Ca^{2+}/CaM. CaM works by binding to and regulating calmodulin binding proteins (CaMBPs). Both apo-CaM and Ca^{2+}/CaM can each bind to a wide variety of calcium-independent and calcium-dependent CaMBPs respectively (Figure 1). The presence of intracellular CaM is well detailed and its diverse functions are widely known (Cohen and Klee, 1988; Vogel, 1994). In contrast, the existence of extracellular CaM (extCaM) has been studied in comparatively few different organisms.

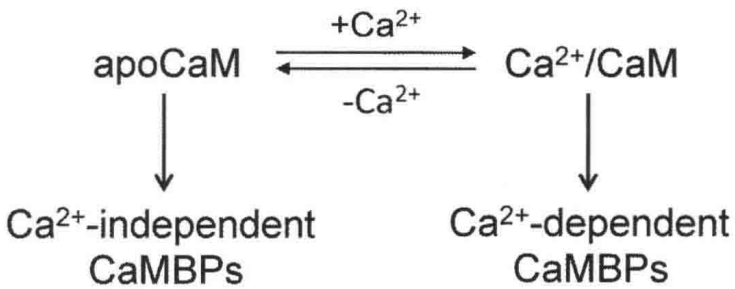

Figure 1. Calcium regulation of CaM and its effect on CaMBP binding.

The presence of extCaM has been conclusively demonstrated in several plant species where it has been shown to regulate cell proliferation (Sun et al, 1994), cell wall regeneration (Sun et al, 1995), gene activity (Zhou et al, 2001), germination (Sun et al, 2009) and stomatal closure (Li et al, 2009). In animals the literature is less focussed but the accumulated data argues strongly that extracellular CaM is a critical signaling component. For example, extCaM mediates nerve regeneration in frogs (Remgard et al, 1995). In hamsters, exogenous CaM amplifies peptide-mediated vasodilation (Ikezaki et al, 1999). Treatment with exogenous CaM stimulates DNA synthesis in rat liver cells (Boynton et al, 1980). In humans, extCaM appears to be required for cell division in the early preimplantation embryo (Woodward et al, 1993) and keratinocytes (Goberdhan et al, 1993). It also increases the proliferation of umbilical vein endothelial cells (Dawson and MacNeil, 1992).

Dictyostelium CaM (DdCaM) is encoded by a single gene (*calA*; DDB_G0279407). A single study indicates that DdCaM knockouts are lethal as they are in other organisms (Clarke, 1990). Intracellular DdCaM has been shown to regulate chemotaxis, development, spore germination as well as protein phosphorylation, among other events in *Dictyostelium* (O'Day, 2003). A large number of calcium-independent and calcium-dependent CaMBPs have been detected but only a handful of them have been identified and characterized (O'Day, 2003; Catalano and O'Day, 2008). As summarized in those reviews, CaMBPs have been

detected in various cellular compartments: cytoplasm (calcineurin, DGAP1, DWWA, myosins, phosphoglycerate kinase, ribosomal protein L19, spectrin/fodrin, thymidine kinase), nucleus (histone H1, nucleomorphin), nucleolus (BAF60a/SMARCD1 homologue, nucleomorphin; Catalano and O'Day, 2012), and the contractile vacuole (VwkA).

CURRENT RESEARCH

Evidence for Extracellular Calmodulin in Dictyostelium

The presence of extracellular CaM (extCaM) in *Dictyostelium* has only recently been verified (O'Day et al, 2012). A proteomic analysis of the extracellular medium indicated the presence of DdCaM as well as the calcium binding protein cbp4a and some putative CaMBPs (Bakthavatsalam and Gomer, 2010). Subsequent work by our group revealed that CaM is secreted throughout growth and development where it regulates several different events. This review will focus on work done to date on extCaM and extCaMBPs in this social amoebozoan and will end with some of the questions, related to their role in signal transduction, that arise from this work.

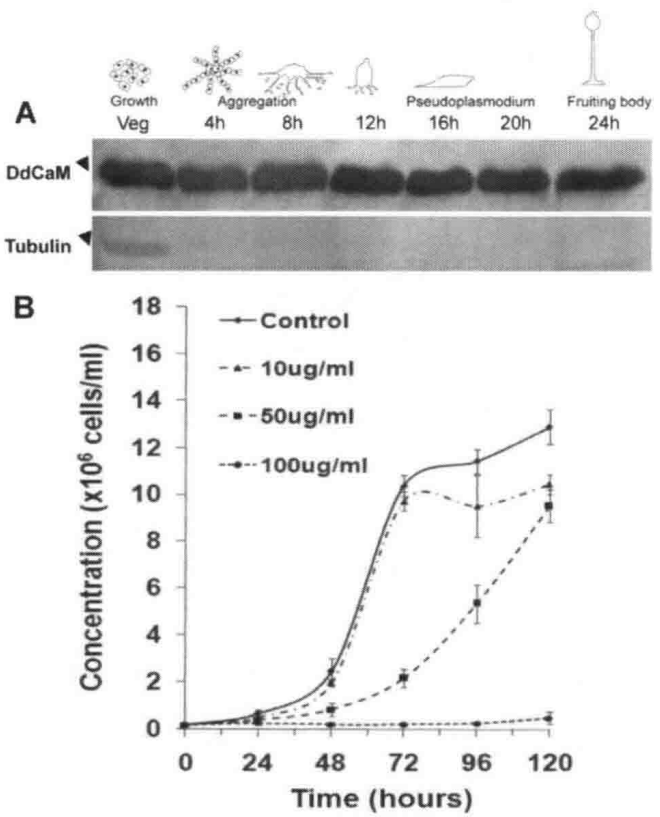

Figure 2. Extracellular CaM expression through growth and development (A) and the effects of exogenous bovine CaM on cell proliferation (B) in *Dictyostelium* (Suarez et al., 2011; O'Day et al, 2012).

Functions of Extracellular CaM during Growth

ExtCaM protein was detected throughout growth and development indicating that it might function at any and all stages (Figure 2, A). To examine the role of extCaM during the growth phase, proliferating cells were treated with different concentrations of bovine CaM (Figure 2,B). Bovine CaM has been show to share high sequence identity with and proven to be functionally identical to *Dictyostelium* CaM (Lydan and O'Day, 1994). The addition of bovine CaM dose-dependently inhibited cell proliferation of cells in axenic cultures with 100ug/ml being almost completely inhibitory. Depending on the organism (Boynton et al, 1980; Dawson and MacNeil, 1992; Goberdhan et al, 1993; Sun et al, 1994; Woodward et al, 1993) those and other results have led to the suggestion that extCaM acts as an autocrine growth factor.

Extracellular CaM Increases cAMP-mediated Chemotaxis

The role of calcium and CaM in mediating chemotaxis has been known for many years mainly through results from pharmacological studies (Gauthier and O'Day, 2001). However, the importance of CaM was attributed to its intracellular locale since extracellular CaM was not known to exist. However, it is now clear that extracellular CaM dose-dependently enhances the rate of cAMP-mediated chemotaxis, providing another function for extCaM in *Dictyostelium* (Figure 3).

Secretion and Processing of CyrA, a CaM-Binding Protein

CyrA was first isolated using the CaM-binding overlay technique (CaMBOT) suggesting that it was a CaMBP (O'Day, 2003). Scanning of the sequence of CyrA further revealed the presence of a putative CaM-binding domain (CaMBD) between amino acids 249 to 275 (Figure 4A). Using CaM-agarose binding and CaM immunoprecipitation, it was subsequently shown that full-length CyrA and the predominant CyrA cleavage products all bind to CaM both intra- and extracellularly (Huber et al, 2012; Suarez et al., 2011).

Another defining aspect of CyrA is the presence of 4 tandem EGF-like (EGFL) repeats in its C-terminus. Detailed and careful studies by Robert Huber and colleagues verified that the first EGFL repeat increases both random cell motility and chemotaxis in *Dictyostelium* via binding to a surface receptor to activate specific downstream signaling events (Huber and O'Day, 2011, 2012a,b,c;).

In total, this work validated CyrA as the first matricellular calmodulin binding protein in a eukaryote (Huber and O'Day, 2012b,c). Developmental analyses showed that CyrA is secreted and processed to release two smaller EGFL repeat-containing polypeptides, CyrA-45 and CyrA-40 (Figure 4).

Bioinformatic analyses had indicated that CyrA was likely secreted since SignalP revealed the presence of a signal peptide in the N-terminus and TargetP revealed the protein is targeted to the secretory pathway (Figure 4).

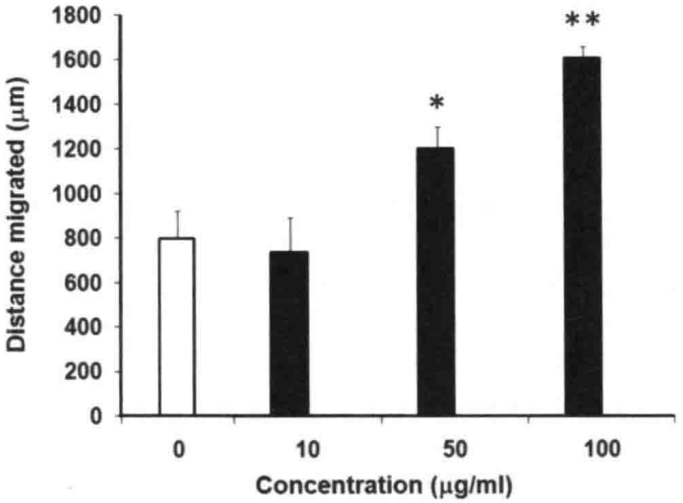

Figure 3. Extracellular CaM and its effect on chemotaxis. Exogenous CaM increases the rate of cAMP-mediated chemotaxis (O'Day et al, 2012). Data presented as the mean distance migrated ± s.e.m (n=4). Mann-Whitney two-sample rank U-test (*p=0.01 vs. 0 μg/ml; **p=0.005 vs. 0 μg/ml).

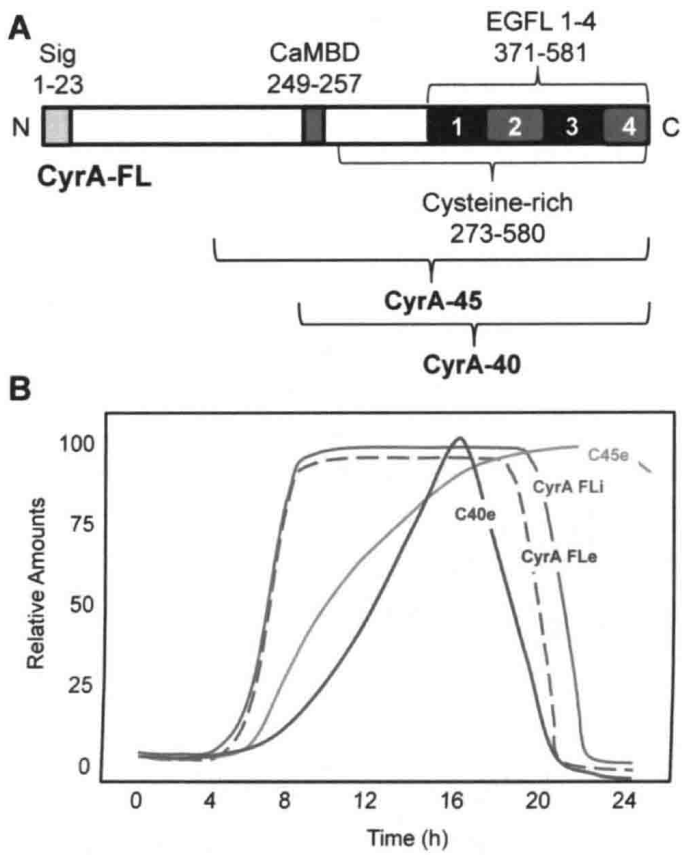

Figure 4. The structure of CyrA and its extracellular processing during development. (A) Full-length CyrA (CyrA-FL) possesses a signal sequence (Sig), a CaMBD and four tandem EGF-like (EGFL) repeats in its C-terminus. The approximate positions of the two major proteolytic products of CyrA of ~45KDa (CyrA-45) and ~40kDa (CyrA-40) are indicated. (B) Full length CyrA is present within the cells (CyrA-FLi) and is temporally secreted (CyrA-FLi). The two major proteolytic products of CyrA CyrA-45 (C45e) and ~40kDa (C40e) are produced during development in different temporal patterns.

Experimental support was provided with the localization of both endogenous CyrA and CyrA-GFP to the ER showing that it is secreted via a conventional secretory pathway (i.e., involving endoplasmic reticulum, Golgi, and vesicle release; Huber et al, 2012). Secretion of the protein itself was verified by western blotting with CyrA being secreted during both growth and development (Figure 4B). A pharmacological analysis further showed that CyrA secretion was dependent on intracellular Ca^{2+} release and active CaM (Huber et al, 2012).

CyrA is thus the first extracellular CaMBP to be experimentally verified in *Dictyostelium* or any other eukaryotic microbe. However, a proteomic analysis did reveal that several putative CaMBPs were present in the extracellular environment (Bakthavatsalam and Gomer, 2010). These include CaMBP15 (DDB0001413) and CaMBP46 (DDB0167501). In addition, puromycin-sensitive aminopeptidase-like protein (DDB0231240) was also found in the extracellular medium. Each of these proteins has been studied in our lab and has been verified as a true CaMBP via CaM-binding and other assays (Catalano and O'Day, 2008; Catalano et al, 2012; O'Day, unpublished).

Both CaM and CyrA Localize to the Extracellular Matrix during Development

During development, a motile multicellular structure known as a pseudoplasmodium is formed that migrates along the substratum in response to light and temperature. Isolation of the extracellular matrix (ECM; called a slime sheath) that surrounds this multicellular tissue, followed by western blotting revealed the presence of CaM, full-length CyrA (CyrA-FL) and CyrA cleavage produces (Figure 5, A; e.g., CyrA-C45, -C40; Huber et al, 2012; Suarez et al., 2011).

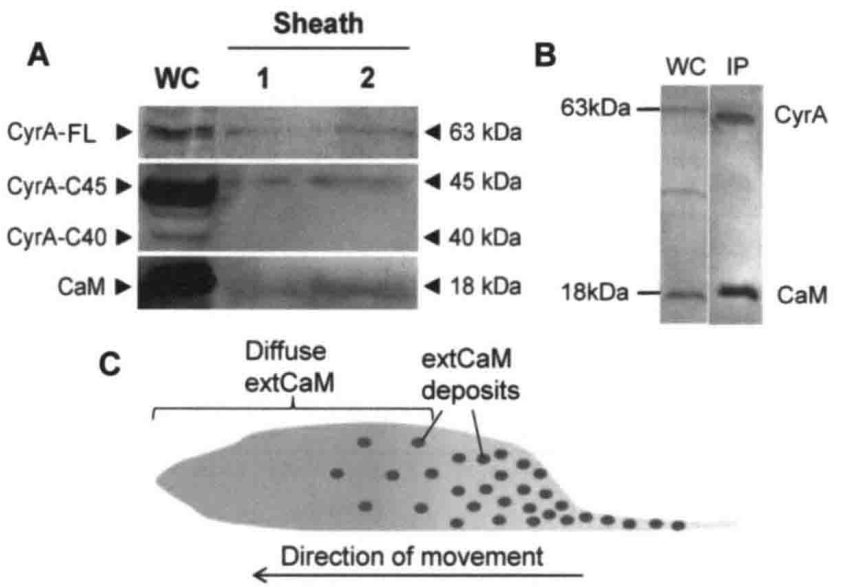

Figure 5. CaM and CyrA in the extracellular matrix. (A) Presence of CyrA and CaM in isolated slime sheath as shown via western blotting. (B) CyrA and CaM are both detected in CaM co-immunoprecipitates. (C) Diagrammatic summary of extCaM localization in the ECM of the pseudoplasmodium (O'Day et al, 2012).

Immunoprecipitation of extracellular CyrA pulled down both CyrA and CaM indicating that they are bound together in the ECM (Figure 5, B; Huber et al, 2012; Suarez et al, 2011). During development, extCaM is diffusely localized throughout the pseudoplasmodium in a gradient that is highest at the anterior end (Figure 5C). This apparently soluble extCaM becomes deposited in insoluble precipitates at the back of the pseudoplasmodium suggesting that the availability of extCaM in the ECM and its effects on CyrA processing and cell chemotaxis will vary along the length of this multicellular stage of development (Suarez et al., 2011; O'Day et al, 2012).

Extracellular CaM Inhibits CyrA Proteolysis

The ability of intracellular CaM to protect proteins from proteolysis has been described in several different organisms (Reddy et al., 2006; Suarez et al., 2011; Zakharov and Mosevitsky, 2007; Zhang et al., 2009). However, the ability of extCaM to similarly protect against proteolysis in the extracellular environment had not been studied. As seen in Figure 6, in the presence of CaM, CyrA proteolysis is inhibited such that the major products (i.e., CyrA-45, -40) are generated at much lower levels (Suarez et al, 2011). To our knowledge, CyrA represents the first extracellular CaMBP whose proteolytic processing is protected by CaM extracellularly.

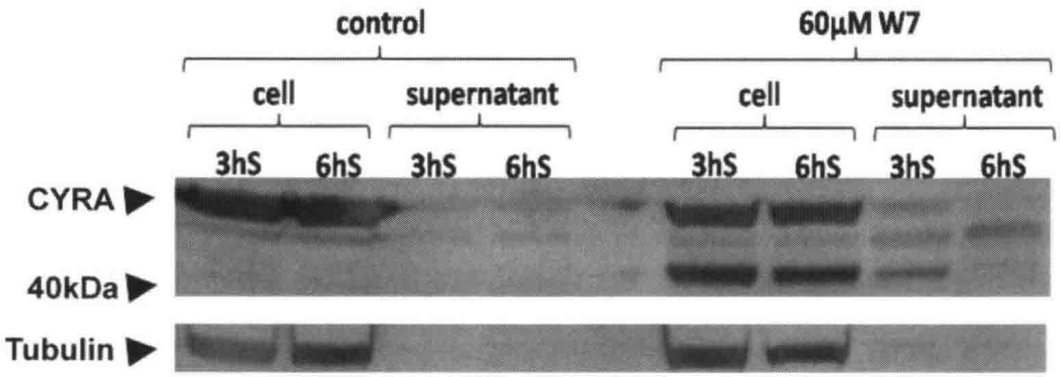

Figure 6. The inhibition of CyrA processing by CaM. Cells were either untreated (left side) or treated (right side) with 60mM of the CaM antagonist W7 after which cells and extracellular medium were isolated and subjected to western blotting (Suarez et al 2011).

CONCLUSION

As summarized in Figure 7, calmodulin serves multiple extracellular functions in *Dictyostelium*. In this chapter, the focus has been on the functions of extracellular calmodulin and its relationship with a secreted CaM-binding, matricellular protein, CyrA.

During growth, CaM is secreted and the extracellular CaM (extCaM) feeds back to inhibit growth. Secretion of CaM continues once cells are starved and embark on multicellular development. During early development extCaM enhances the chemotactic