THE ACTIVITY OF CORONIN A IS DEPENDENT ON A PROTEIN(S) SECRETED BY *DICTYOSTELIUM DISCOIDEUM* IN THE EARLY STARVATION RESPONSE

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ABSTRACT

The social amoebae *Dictyostelium discoideum* is used widely as a model organism for studying development. It becomes chemotactic to autocrine signals following starvation, initiating a cell aggregation program in which different multicellular behaviors can be studied to elucidate mechanisms involved in development of eukaryotic cells. Starvation induces cell aggregation that plays an essential role in multicellular morphogenesis of the slime mold *Dictyostelium discoideum*. While secreted factors released by *D. discoideum* in the medium are known to regulate the developmental cycle in this amoebae have been identified, the downstream molecular signaling mechanisms involved in the transition from unicellularity to multicellularity remain largely uncharacterized. Our previous work suggests that cell aggregation is driven by coronin A that is required for *D. discoideum* to initiate the early development by activating the cAMP signaling pathway that in turn is essential for upregulation of genes involved in aggregation and development. In contrast to wild-type cells, cells lacking coronin A resulted in *D. discoideum* strains that undergo abnormal morphogenesis when incubated with factors from the starvation conditioned medium. These previous results suggest that the activity of coronin A is dependent on a specific factor(s) secreted by starving cells that regulate the transition from the nutrient deprivation to development stage. However, how coronin A regulates the organization of initial development, and which of these secreted factors is responsible for the activation of coronin A is not known. To characterize, purify, and identify the specific factor(s) required for coronin A-dependent development, heat-sensitivity, proteinase K, and phospholipase D treatments were performed. Our results implicate

proteins as the biochemical entity inducing morphogenesis, which are heat-labile and protease-sensitive factors. Furthermore, ultrafiltration of conditioned medium suggested that the active fraction consists of a protein or protein complex with a molecular weight above 100.000 Dalton. We have further defined the properties of the active fraction as well as initiated purification of the protein(s). We have utilized ion-exchange chromatography, and were able to separate the activity into discrete fractions. In an attempt to screen candidates in these fractions for development-inducible factors, we compared cell aggregation activities in wild-type strains DH1-10 and coronin A-deficient *Dictyostelium* discoideum cells after further separation of the proteins by size exclusion chromatography. Our data suggested evidence for the requirement of a coronin A-dependent factor(s) in the cell aggregation process. In addition, our work revealed that ion exchange and gel filtration chromatography are suitable methods for characterizing the factors. Together, this study may contribute to a better understanding of the secreted factor(s), and their signaling pathways, and define the basis for regulatory mechanisms involved in the early starvation response of *D. discoideum.*

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1. INTRODUCTION

Introduction on the developmental life cycle of social amoebae *Dictyostelium discoideum*

Dictyostelium discoideum is a social amoeba that feeds on soil bacteria, which are present in decomposing vegetation, through phagocytosis in order to maintain its mitotic division [Loomis, 1975; Robertson & Grutsch, 1981; Clarke, Yang & Kayman, 1988; Insall, 2005; Mahadeo & Parent, 2006; Romeralo et al., 2011]. Normally, under high nutrient environments, *D. discoideum* amoeboids proliferate as individual cells [Mahadeo & Parent, 2006]. However, when bacterial nutrient sources become depleted it causes individual cells to starve [Weijer, 1999; Mahadeo & Parent, 2006]. Starving cells stop dividing by binary fission, and up to a million cells undergo cell aggregation [Mahadeo & Parent, 2006; Weijer, 2009]. Cell aggregation is important for allowing *D. discoideum* to initiate a development program leading from unicellularity to a multicellular organism comprised of the fruiting body-containing spores, which is essential for long survival through the nutrient-depleted environments (see also figure 1 below) [Kishi et al., 1998; Mahadeo & Parent, 2006; Wang et al., 2011; Dubravcic et al., 2014]. Under starving conditions, cells trigger downregulation of the genes associated with growth, whereas genes essential for development that leads to a multicellular state are expressed [Mehdy and Firtel, 1985; Insall et al, 1994; Chen et al., 1996; Iranfar et al., 2003; Loomis, 2014]. Cell aggregation is characterized by progression into early development after 4 to 6 hours of starvation [Mehdy and Firtel, 1985], and accompanying secretion of 3'-5'-cyclic adenosine monophosphate (cAMP) [Insall et al., 1994]. In starving cells, early

developmental genes for the cAMP receptor (cAR1) and adenylyl cyclase A (ACA) in the cAMP signal transduction pathway are induced [Insall et al., 1994].

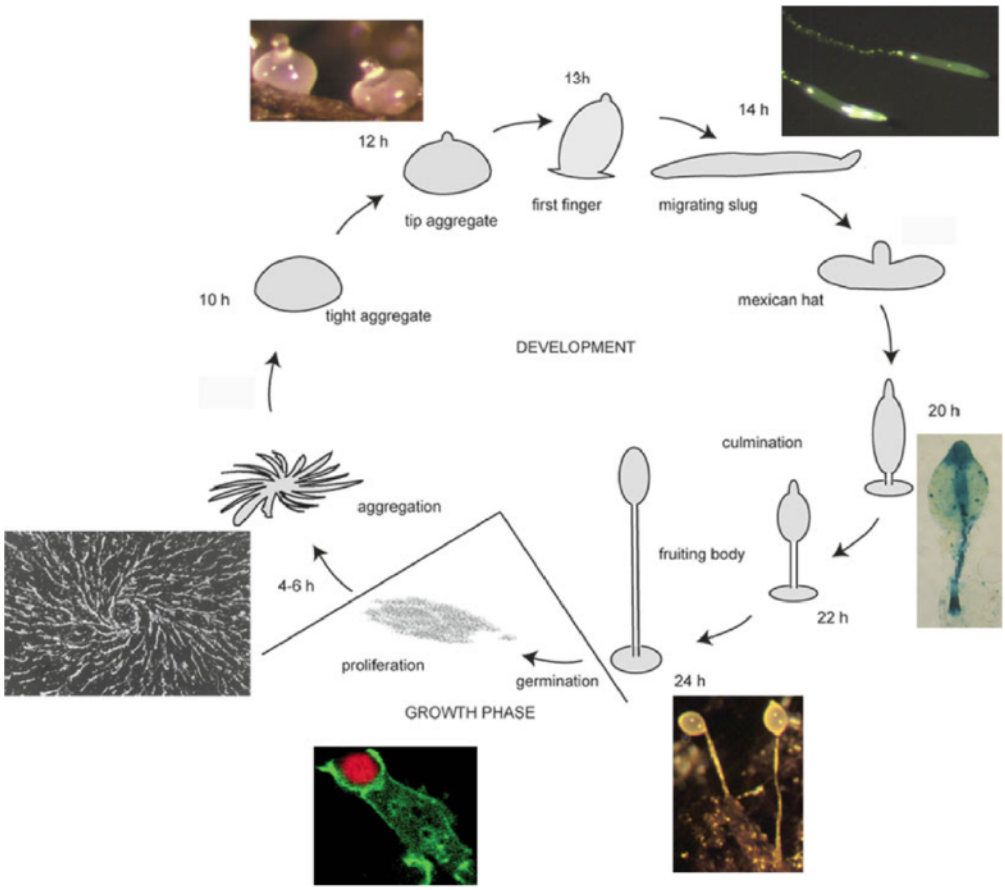


Fig. 1 illustrates the life cycle of *Dictyostelium discoideum*. The life cycle contains two different stages: growth and development. During the presence of bacterial nutrients, *D. discoideum* grow as individual cells. When starved, single *D. discoideum* cells do not proliferate mitotically by binary fission, and initiate cell aggregation, which is followed by a formation of migratory slug that enters into the complete development, the fruiting body-containing spores. Illustration retrieved from Eichinger & Rivero (2013).

Once the cells initiate the cAMP signaling pathway by secreting and releasing cAMP in a wave-like manner, they aggregate to form what is called an aggregation center that stimulates the production of more cAMP by neighboring cells that become chemotactic to cAMP [Weijer, 1999; Mahadeo & Parent, 2006; Swaney et al., 2010;

Schaap, 2011]. See the figure 2 below. As the cAMP gradient reaches a high enough level, it activates a main intracellular signaling component such as the cAMP-dependent protein kinase A (PKA) in pre-aggregating cells [Taylor et al., 1990; Loomis, 1998; Brown & Firtel, 1999; Chisholm & Firtel, 2004; Schaap, 2011]. PKA induces the expression of developmental genes that are required for cell aggregation, such as those that encode the cAMP receptor (cAR1) and adenylyl cyclase A (ACA) [Schulkes & Schaap, 1995]. The latter, which is expressed at maximal levels during starvation-induced cell aggregation, accumulates further production of cAMP to a level that results in

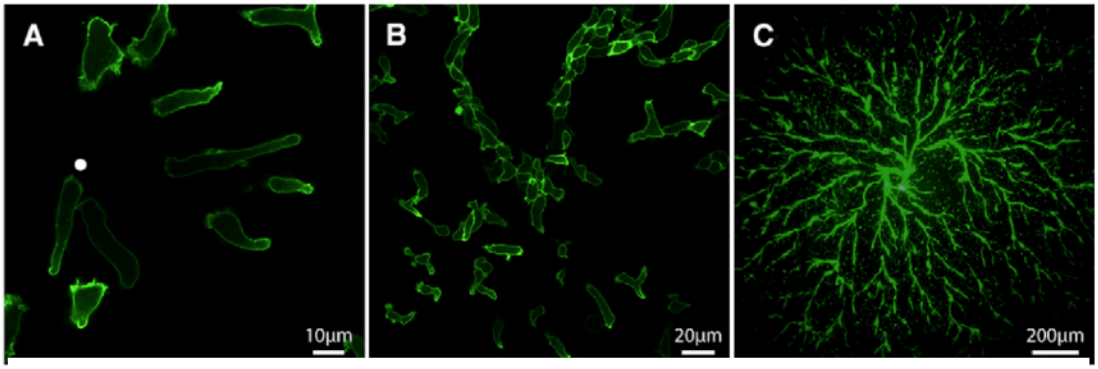


Fig. 2 illustrates chemotaxis and cell aggregation of *D. discoideum*. **A**. Chemotaxis of *D. discoideum* towards 3'-5'-cyclic adenosine monophosphate (cAMP), as shown by white dot. **B**. Initiation of cell aggregation induced by starvation. **C**. Starving cells aggregate at the center of cell aggregation. Figure retrieved from Fets, Kay, & Velasquez (2010).

synthesis of more internal cAMP molecules [Gerisch et al., 1979; Dinauer et al., 1980; Iranfar et al., 2003; Kriebel & Parent, 2004; Matsuoka et al., 2004; Loomis, 2014]. This transduction process is known as the cAMP relay that allows the internal cAMP signal to be amplified as waves, in which endogenous cAMP diffuses from each *D. discoideum* cell as an extracellular cAMP chemoattractant [Matsuoka et al., 2004; Loomis, 2014]. This cAMP then binds to the cAMP receptors on neighboring cells by local diffusion [Malchow & Gerisch, 1973; Mahadeo & Parent, 2006]. Figure 3 illustrates an example of

neighboring cells sensing cAMP released by a group of developed cells during the cAMP relay. The cAMP relay continues to secrete cAMP that diffuses out from a group of pacemaker cells, and then neighboring cells, resulting in cAMP pulses that can be emitted from the cAMP signaling aggregation center, and spread through the *D. discoideum* population [Roos et al., 1975; Mahadeo & Parent, 2006].

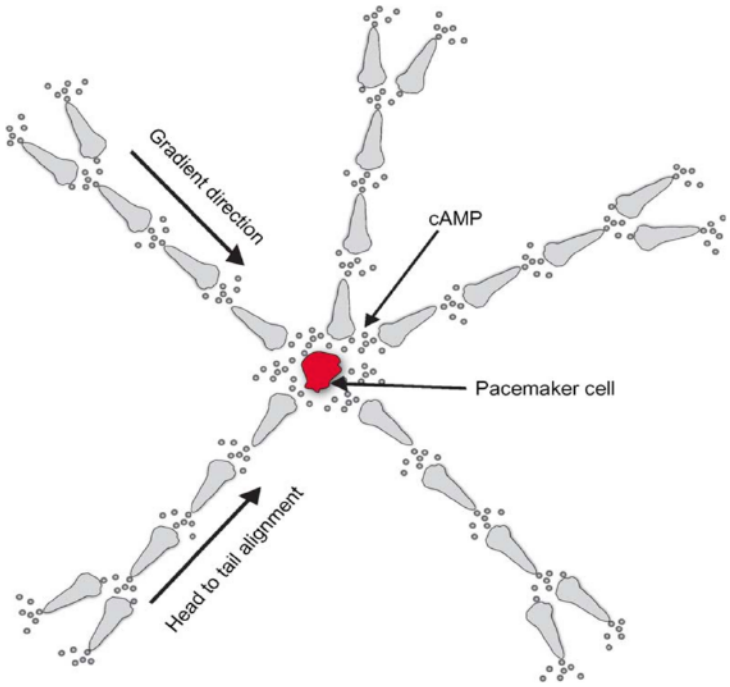


Fig. 3. Neighboring *D. discoideum* cells sense cAMP released by a cell known as a "pacemaker cell" during the cAMP relay. Cells move towards the cAMP gradient through chemotaxis in a head-to-tail manner. Figure retrieved from Mahadeo& Parent, 2004.

The hallmark of the cAMP relay is that it amplifies cAMP waves in a paracrine manner, in which neighboring cells express more cAR1 receptors and ACA enzymes by binding to the cAMP chemoattractants [Theibert & Devreotes, 1986; Siegert & Weijer, 1989; Insall et al., 1994; Mahadeo & Parent, 2006; Weijer, 2009]. As a result of cAMP waves, cells move towards a higher concentration of cAMP by chemotaxis, and gather locally at

the cAMP signaling center to aggregate into a motile slug-shaped structure, typically after 4 to 6 hours of complete cell aggregation [Siegert & Weijer, 1989; Bagorda et al., 2006; Mahadeo & Parent, 2006; Loomis, 2008; Eichinger & Rivero, 2013]. When slug formation is completed, this multicellular organism, while at the same time relaying cAMP chemoattractant waves, ultimately develops into the fruiting body-containing spores [Siegert & Weijer, 1989; Kishi et al., 1998; Dormann & Weijer, 2001; Kimmel & Firtel, 2004; Mahadeo & Parent, 2006].

Whereas the cAMP relay as well as the downstream activation cascade is well characterized, how *D. discoideum* cells initiate the cAMP relay upon starvation is less well understood. Early co-workers have pointed to a potential role of a well-known secreted factor termed conditioned medium factor (CMF) as a developmental regulator of this cAMP relay required for early development [Gomer et al. 1991; Yuen et al., 1991; Jain & Gomer, 1994]. This factor induces cell aggregation by binding to its specific receptor, conditioned medium factor receptor 1 (CMFR1), when cells at a high density starve [Gomer et al. 1991; Yuen et al., 1991; Jain & Gomer, 1994]. Following receptor activation, CMFR1 activates the cAMP relay via activation of adenylyl cyclase downstream the G-coupled cAMP receptor 1 (cAR1) [Gomer et al. 1991; Yuen et al., 1991; Jain & Gomer, 1994; Yuen et al., 1995]. As shown by Iijima and co-workers, CMF^{450} is another secreted factor composed of a complex that has developmental activity similar to that observed on CMF, promoting morphogenesis, as well as differentiation in starving cells; however, it is not known whether CMF^{450} participates in regulating the cAMP relay [Gomer et al. 1991; Yuen et al., 1991; Jain & Gomer, 1994; Iijima et al., 1995]. Having described briefly the role of CMF in early development, the activity of

CMF is essential for the initiation of cAMP relay, but is insufficient for activation of adenylyl cyclase during starvation in *D. discoideum*, the evidence for which is discussed in chapter 2. On the other hand, the initiation of cAMP relay is dependent on CMF, however, it is not clear how cAMP production is first initiated.

Recent work has uncovered an important role for coronin A in the activation of early development in *Dictyostelium discoideum* [Vinet et al., 2014].

D. discoideum coronin A belongs to the coronin protein family, which is evolutionarily conserved in all eukaryotic organisms analyzed to date [Eckert et al., 2011]. Coronin proteins are characterized by the presence of a central WD (tryptophan-aspartic acid) repeats forming a β-propeller, which is followed by a C-terminal coiled-coil domain responsible for oligomerization [de Hostos et al., 1991; de Hostos et al., 1993; de Hostos et al., 1999]. Structural and bioinformatics analyses demonstrate amino acid sequence homology of coronin molecules with the β subunits of trimeric G proteins, and report that mammalian coronin 1, which is related to this *D. discoideum* coronin protein, shares a structure of seven bladed-β-propeller with that of the Gβ subunit [de Hostos et al., 1991; Gatfield et al., 2005; Appleton et al., 2006]. Interestingly, in neurons, coronin 1 was recently shown to activate the cAMP-dependent kinase signaling transduction that has been implicated in cognition and behavior [Jayachandran et al., 2014]. Our recently published work suggests that in *Dictyostelium discoideum*, upon starvation, the coronin A homologue member (*D. discoideum* also expresses a second isoform of coronin, coronin B, that consists of a tandem coronin molecule lacking the coiled-coil domain, which appears not to be involved in development [Shina et al., 2011]) also plays an essential role in initiating the cAMP-dependent kinase signaling pathway

(cAMP relay), thereby inducing cAMP chemotaxis that leads to cell aggregation, which is followed by subsequent development needed for *D. discoideum* multicellularity [Vinet et al., 2014].

To date, most coronin molecules are expressed throughout the eukaryotic kingdom, from fungi, protists, to all eukaryotes, in which every organism contains at least one coronin isoform [Eckert et al., 2011]. Coronin A was first identified as a protein co-purifying within the actin/myosin complex in *Dictyostelium discoideum*, and based on this as well as the observations that this organism lacking coronin A revealed aberrant phagocytosis, cytokinesis, and chemotaxis [de Hostos et al., 1991; de Hostos et al., 1993; Neer et al., 1994; Shina et al., 2011]. As a result, coronin A, as well as its homologues in other organisms, was labeled as an F-actin interacting and modulating protein. However, more recent work suggests that the defects in phagocytosis and chemotaxis are not due to a role for *D. discoideum* coronin A in F-actin modulation [Vinet et al., 2014]; in addition, coronin protein distribution analyses do not show changes in F-actin arrangement upon genetic deletion of other coronin molecules [Jayachandran et al., 2008; Mueller et al., 2011; Pieters et al., 2013; Salamun et al., 2014].

As described previously, coronin A in *Dictyostelium discoideum* initiates the cAMP relay during the onset of starvation. Disruption of *corA* gene results in amoeba that are unable to reach the cell aggregation stage, resulting in abnormal morphogenesis [Vinet et al., 2014]. The deletion of *corA* gene impacts early stages of development and later stages, namely, the ability of cells to induce expression of developmental genes associated with initiation of the cAMP relay, to trigger cell aggregation, to form a

migratory slug, and to enter into the final developmental stage to produce the fruiting body-containing spores [Vinet et al., 2014].

Previous studies using exogenous cAMP pulses show that starved *D. discoideum* mutants lacking coronin A are rescued in their ability to produce cAMP required for cAMP chemotaxis by up-regulating early gene expression [Vinet et al., 2014]. This work suggests that coronin A functions upstream of the cAMP signaling transduction to sense a developmental factor(s) from the conditioned medium (CM) to initiate cAMP pulses essential for cell aggregation [Vinet et al., 2014]. Although our results suggest that the coronin A-dependent cAMP cascade activation is part of the developmental signaling pathway, the molecular details leading to activation of coronin A that initiates the cAMP signaling pathway still need to be delineated, and secreted factors inducing the coronin A-dependent cell aggregation signaling pathway have not been identified. This study focuses on defining the underlying molecular mechanisms of a CM upstream factor(s) dependent on coronin A for cell aggregation. Additionally, the aim of this research was to isolate, purify, and characterize secreted factors in CM by heat, proteinase K, and phospholipase D treatments, including by ammonium sulfate precipitation, and chromatographic methods. This research addressed our hypothesis that coronin A required a cell-aggregation factor for initiation of the earliest development in *D. discoideum* in response to starvation. It was found from our heat sensitivity, proteinase K, phospholipase D, and chromatographic analyses that the ability of coronin A to relay a cell aggregation signal to early gene expression (cAMP relay) was likely dependent on a CM protein that functioned as an upstream factor, playing an early role in development.

This study will provide insights into properties, and functions of the active factor(s) involved in the cell aggregation signaling pathway.

The context of this thesis is as follows: in chapter 2 we summarize the evidence that support the involvement of coronin A in regulating *D. discoideum* morphogenesis, and demonstrate that targeted deletion of the *corA* gene impairs the ability of *D. discoideum* to aggregate into the multicellular structure.

In chapter 3, we describe the *Dictyostelium discoideum* cell culture and conditioned medium (CM) preparation methods. Then, we describe cell aggregation assays (conditioned medium assays) that were used to examine early and late morphogenesis of wild-type *D. discoideum* cells and/or coronin A-deficient cells; from the starvation, cell aggregation (4 to 8 h), to fruiting body stage (16 h). Next, we examine the heat sensitivity, proteinase K, and phospholipase D analysis that characterized our active components in CM as follows: populations of *D. discoideum* cells were transferred to conditioned medium, where they were exposed to different developmental factors. Following developmental induction, we characterized factors by monitoring the morphology of the cells. In addition to this biochemical characterization of factors, ammonium sulfate precipitation, ion exchange, and size exclusion chromatography methods were used to purify, and fractionate secreted CM factors into homogeneity, and active fractions, which will be tested for cell aggregation activities in the wild-type *D. discoideum* cells by analogy with *corA*$^-$ mutants.

In chapter 4, we describe experiments related to the biological role of an active secreted factor(s) from the conditioned medium (CM) on starving cells. These experiments allowed us to characterize a fraction isolated in the conditioned medium that

is used by *D. discoideum* cells to induce cell aggregation. In addition, in characterizing coronin A-dependent factors, we present evidence that most secreted factors are heat-, protease-sensitive, and composed of proteins that possess a cell aggregation-inducing property, which promotes the coronin-regulated cAMP signaling pathway [Vinet et al., 2014]. Lastly, in chapter 5, we conclude this thesis with a general discussion.

In this study we have focused on the biochemical characterization, purification, and isolation of active factors from the conditioned medium (CM) secreted by *Dictyostelium discoideum* during the starvation response. CM is known to contain several secreted factors that are indispensable for a wide range of developmental processes in *D. discoideum* [Loomis, 2014]. Our findings described in this thesis suggest that coronin A is required for sensing a specific-secreted factor(s) present in CM for initiation of cell aggregation in response to the starvation.

2. LITERATURE REVIEW

Dictyostelium discoideum coronin A

Coronin A was initially extracted from actin-myosin complexes in *Dictyostelium discoideum* as a co-purifying protein with an estimated molecular mass of 55 kDa [de Hostos et al., 1991; de Hostos et al., 1993]. Coronin A was named after its specific localization to projections present in the cell cortex within the crown-shaped structure [de Hostos et al., 1991; de Hostos et al., 1993]. In the aggregation stage, coronin A molecules were found to be predominantly accumulated in the front of migrating cells [de Hostos et al., 1991]. Interestingly, while earlier studies on the cAMP signaling pathway following the deletion of actin binding proteins, such as α-actinin, gelation factor, and severin, revealed that they did not alter aggregating behavior, or cAMP chemotaxis, whereas numerous studies demonstrated that the absence of coronin A impaired the ability of cells to chemotax, or initiate a developmental stage [Wallraff, et al., 1986; André et al., 1989; Brink et al., 1990; de Hostos, et al., 1991; Shina et al. 2011; Vinet et al., 2014]. For instance, Shina et al. (2011) showed that after cAMP stimulation, *corA*$^{-}$ mutants in *D. discoideum* strains exhibited defects in phagocytosis of bacteria, chemotaxis, or development which we also observed in our current experiments using conditioned medium (CM) [unpublished data; Vinet et al., 2014]. These studies suggest that coronin A protein has a significant role in activating a developmental signaling cascade that regulates a cytoskeletal structure [de Hostos, 1999; Nakamura et al., 1999; Noegel & Schleicher, 2000; Shina et al., 2011].

As described before, coronin A is characterized by a WD (Tryp-Asp) repeat motif that might be utilized for interactions with other proteins, thus forming multiprotein

complexes in order to facilitate cell mobility, phagocytosis, and cytokinesis in *D. discoideum* [Neer et al., 1994; Shina et al., 2011]. Furthermore, besides sharing the polypeptide sequence homology with the β subunits of the trimeric G proteins, due to the tendency of coronin A to contain a carboxyl-terminal domain that folds into an α-helical secondary structure, this C-terminal domain was proposed to be essential for binding to actin filaments that may serve to promote a downstream developmental signaling transduction pathway indispensable for cytokinesis, endocytosis, phagocytosis, cell mobility, chemotaxis, and development [Zot & Potter, 1987; de Hostos, 1991; Noegel & Schleicher, 2000]. However, as mentioned in chapter 1, it has recently become evident that coronin A regulate the cAMP signaling pathway in an F-actin independent manner [Vinet et al., 2014].

A regulatory role of coronin A in cell aggregation in *D. discoideum*

Coronin A functions as a regulator of developmental signaling pathways in *D. discoideum*. Previous work has indicated that mutants lacking coronin A failed to sense a secreted factor(s) from the conditioned medium (CM), and did not initiate their developmental cycle [Vinet et al., 2014]. This phenotype could be restored by re-expression of coronin A in a complemented strain [Vinet et al., 2014]. Moreover, upon addition of external cAMP to the *corA*$^-$ mutants, the cells recovered their ability to induce cell aggregation, as well as subsequent differentiation into the fruiting spores resistant to nutrient depletion [Vinet et al., 2014]. This work suggests that the cAMP-treated mutants adopt the phenotype of the wild-type cells, and this can be explained as follows: 1) coronin A activation or regulation per se is not needed for subsequent development

beyond the cAMP signaling pathway, but the presence of coronin A at the membrane projections may be apparently indispensable for initiation of the starvation-induced cAMP relay leading to cell aggregation [de Hostos et al., 1991; de Hostos et al., 1993; de Hostos; 1999; Vinet et al., 2014].

Given that the coronin A-deficient cells do not respond to the developmental signals derived from the conditioned medium (CM) [Vinet et al., 2014], and this medium contains active factors regulating *D. discoideum* morphogenesis [Loomis, 2014], further investigation of these functional and biochemical molecules in the medium is crucial to understanding how cell aggregation requires cells to communicate with each other in order to develop into multicellular organisms.

Factors secreted by *D. discoideum* inducing early development are dependent on coronin A

One of the important mechanisms for early development in *Dictyostelium discoideum* is the binding of a molecule to a surface receptor that results in relaying a signal to a specific effector component that controls developmental gene expression [Loomis, 2014]. This regulatory molecule that drives the multicellular developmental program via cell aggregation in response to starvation conditions can be composed of nucleic acids, amino acids, proteins, lipids, steroids, or polylketides [Maeda & Kuwayana, 2000; Loomis, 2014]. A variety of these molecules is secreted into the conditioned medium (CM) that is observed when cells are subjected to depletion of nutrients [Mehdy & Firtel, 1985; Maeda & Kuwayana, 2000; Oohata et al., 2009; Vinet et al., 2014], and may have roles in the developmental processes (Figure 4) [Loomis, 2014].

As described above, mutants lacking coronin A did not respond to developmental signals by secreted factors. Given that currently we do not know which type of conditioned medium molecule is responsible for activating coronin A that initiates the cAMP relay [Vinet et al., 2014], in this thesis we aim to define the underlying molecular mechanisms of a diffusible coronin A-dependent factor(s), and learn more about its biochemical and biological property, e.g., whether this candidate factor is classified as a lipid, nucleic acid, or protein that exhibits the cell aggregation-inducing property. In particular, we seek to determine whether the cell aggregation activity of coronin A is dependent on this property. As such, use of the conditioned medium (CM) is the starting point for investigation of the coronin A-dependent mechanisms of regulation in the *D. discoideum* development, and whether an active factor influences developmental processes specifically mediated by the coronin A protein [Maeda & Kuwayana, 2000; Oohata et al., 2009; Loomis, 2014; Vinet et al., 2014].

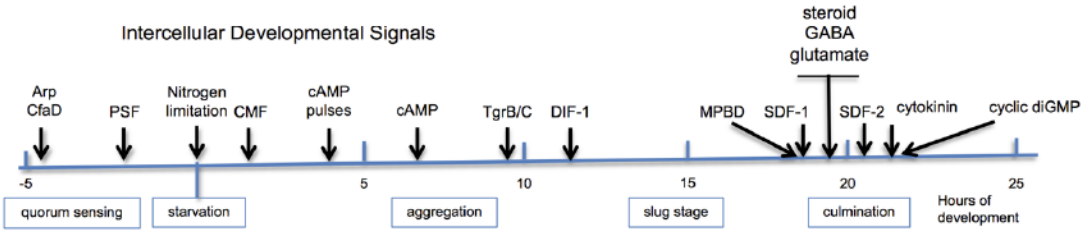


Fig. 4. Signaling molecules have regulatory roles in development of *D. discoideum* during the early starvation response. The abundance of different molecules in the conditioned medium (CM) can vary widely. Figure retrieved from Loomis, 2014.

Coronin A-deficient cells exhibit a normal prestarvation response, and are dependent on a certain cell aggregation-inducible factor in the conditioned medium

Studies by Burdine and Clarke (1995) demonstrated that during the vegetative growth *D. discoideum* produced a glycoprotein that regulated genes associated with early development. This factor is called prestarvation factor, also known as PSF, and is responsible for upregulation of a gene encoding discoidin-I whose activation is essential for both the cAMP signaling pathway, and cell aggregation in response to early starvation [Burdine & Clarke, 1995; Mahadeo & Parent, 2006; Loomis, 2014].

At the unicellular stage of growth, *D. discoideum* does not undergo cell aggregation, a stage during which the population of individual cells feeds on soil bacteria that secretes folic acid [Burdine & Clarke, 1995; Mahadeo & Parent, 2006; Bagorda & Parent, 2008]. Similar to the role of cAMP molecules in attracting *D. discoideum* toward the cAMP gradient, folic acid functions as a chemoattractant which if produced at high levels, keeps growing cells chemotactic to this bacterial nutrient [Pan et al., 1972; Clarke et al., 1987; Manahan et al., 2004; Mahadeo & Parent, 2006]. As more folic acid chemoattractants are released into the extracellular medium by bacteria present at a high concentration, they inhibit the activity of the prestarvation factor (PSF), secreted by *D. discoideum* cells [Rathi et al., 1991; Clarke & Gomer, 1995; Mahadeo & Parent, 2006]. When the growth capability of bacteria reduces, early starvation response is accompanied by an increase in PSF signaling that allows cells to develop by expressing discoidin-I, and sensing cAMP [Berger et al., 1985; Rathi et al., 1991; Mahadeo & Parent, 2006; Loomis, 2014].

In exploring whether coronin A was required for PSF signaling in the early starvation response of *D. discoideum*, Vinet et al. (2014) used western blot analysis, and found that wild-type (DH1-10 *D. discoideum*), and *corA*$^{-}$ mutants exhibited a similar

increase in discoidin-I levels when they were treated with decreasing concentration of bacteria. In addition, using folic acid assays and western blot analysis, Vinet et al. (2014) demonstrated a similar decrease in gene expression of discoidin-I in wild-type cells and *corA*$^-$ cells, as well as a similar increase in cAMP levels after measuring cAMP production. This suggests that in the absence of coronin A these mutants were able to respond to PSF signal. These results conclude that the function of coronin A is not required for regulating discoidin-I expression.

Activation of developmental gene expression depends on the presence of coronin A

In *Dictyostelium discoideum*, there are heterotrimeric G proteins coupled to the cAMP receptors, and they are known to be composed of α, β, and γ subunits [Firtel, 1996; Omosigho et al., 2014]. The associated Gαβγ subunits that remain physically bound to the cAMP receptors result in an inactivated complex [Omosigho et al., 2014]. Up-regulating synthesis of cAMP that promotes the starvation-induced cAMP relay, chemotaxis, and cell aggregation requires hydrolysis of Gβγ subunits from the Gα subunit [Kumagai et al., 1989; Omosigho et al., 2014]. Activation of the cAMP G protein-coupled receptor by cAMP allows guanosine diphosphate (GDP) to be dissociated from the Gα subunit [Sprang, 1997; Mahadeo & Parent, 2006; Weijer, 2009]. Then, as guanosine triphosphate (GTP) binds to this subunit, the Gα unit dissociates from the β-γ complex of G protein [Sprang, 1997]. During the course of dissociation, the Gβγ complex, and Gα subunit at the plasma membrane are functionally different in terms of mediators they regulate. The Gα subunit, particularly the Gα2 component coupled to the

cAMP receptors 1 (cAR1) [Mahadeo & Parent, 2006], which is one of the twelve Gα subunits harbored by *D. discoideum*, was shown to regulate the activity of adenylyl cyclase A (ACA) while the Gβγ dimer targeted different signaling components such as ion channels, phospholipase CB (PLCβ), and another isoform of adenylate cyclase [Kumagai et al., 1989; Wu et al., 1995; Omosigho et al., 2014].

Kumagai et al. (1989) were able to show that Gα2 subunit expression was upregulated by starvation-inducible cAMP pulses, resulting in the formation of cell aggregation. In the aggregation stage, the mechanism of Gα2 in *D. discoideum* was found to be essential for the cAMP relay, which leads to secretion of more cAMP molecules [Kumagai et al., 1989; Okaichi et al., 1992]. During starvation, Gα2 was significantly expressed in the wild-type cells, but barely in the *corA*$^-$ mutants [Vinet et al., 2014]. A possible explanation for this was suggested: coronin A functions upstream of the Gα2 subunit [Vinet et al., 2014]. Among the early developmental genes needed for entry into the aggregation stage are *carA* (cAMP receptors), *aca* (adenylyl cyclase A), and *csA* (development marker contact site A, also known as gp80, or cell adhesion molecule) [Iranfar et al., 2003; Loomis, 2014]. Quantitative PCR analysis of mRNAs indicated that following starvation stimulation the developmental genes were expressed normally in the wild-type cells as opposed to the coronin A-deficient cells displaying at least or no detectable *carA*, *aca*, and *csA* mRNAs [Vinet et al., 2014]. In addition to reduction of *csA* mRNA levels, Vinet et al. (2014) further assessed by western blot protein levels of contact site A protein; *csA*, is known to be one of the genes most highly regulated by cAMP on the onset of cell aggregation [Faix et al., 1992], and coronin A deletion did not result in *csA* expression [Vinet et al., 2014]. Altogether, from these data with the findings

described above, it was suggested that morphological changes needed for early development may be originally the result of the activation of coronin A by a specific conditioned medium factor, stimulating the cAMP signaling pathway (cAMP relay) involving the Gα2βγ subunits that regulate the expression of the developmental genes [Vinet et al., 2014].

3. MATERIALS AND METHODS

Dictyostelium discoideum cell growth

DH1-10 *D. discoideum* cells, which were received as a kind gift from the lab of Pierre Cosson at the University of Geneva, were axenically cultured at 21 °C in flasks containing autoclaved HL5 (40 g/8L Bacto™ Proteose Peptone No. 3, 40 g/8L Bacto™ Proteose Peptone, 40 g/8L Bacto™ Yeast Extract, 0.056 M dextrose, 0.0013 M $Na_2HPO_4 \cdot 2H_2O$, 0.0025 M KH_2PO_4, pH 6.6) media in the range from 0.2 x 10^6 to 2 x 10^6 cells/ml. Penicillin-streptomycin (300 ug/ml) supplemented to the HL5 media was used as the antibiotics [adapted from Watts and Ashworth, 1970; Vinet et al., 2014]. Strains with coronin A deletion made from the DH1-10 strains in collaboration with Anna Dardel from the lab of Pierre Cosson were axenically cultured in same HL5 media at 21 °C from 2 x 10^5 to 9 x 10^5 cells/ml [Vinet et al., 2014]. Both DH1-10 and coronin A-deficient strains in HL5 media were cultured with shaking at 160 RPM overnight. Wild-type and coronin A-deficient cells never reached more than their cell density of 2 x 10^6 and 9 x 10^5 cells/ml, as measured in 1:10 dilution by a hemocytometer under a microscope. Cells were passaged into 20 to 100 mL fresh HL5 media daily. In order to ensure that over time the cells do not become senescent or compensate for the deletion of coronin A, fresh cells were thawed from frozen stock (-80° C) twice a month.

Starvation and conditioned medium (CM) sample preparation

Fresh conditioned medium (CM) was prepared and harvested by following the method in Vinet et al. (2014). DH1-10 *D. discoideum* cells proliferated in 4 liters of the axenic HL5 media were transferred into Sorvall™ RC 6 Plus centrifuge tubes (Thermo

Scientific), and were collected by centrifugation at 400*xg* for four minutes at 4°C. The supernatant was discarded and the cell pellets were washed with nutrient-free buffer, PBM (0.02 M KH_2PO_4, 10 μM $CaCl_2$, and 1 mM $MgCl_2$, pH 6.2). The cells (pellets) were starved by washing twice with PBM, resuspensed in PBM at a cell density of 1.0 x 10^7 cells/ml. The cells were shaken at 110 RPM for eight hours at 22°C in order to induce secretion of developmental factors into the starvation-conditioned medium (CM) in the supernatant. After eight hours, the starved cells were discarded, and the supernatant that was stored as CM was clarified by centrifugation at 400*xg* for five minutes at 4°C. CM was harvested by centrifugation at 10,000*xg* for 15 minutes at 4°C, filtered through a 0.45 μm filter, aliquoted in liquid nitrogen, and stored at - 80°C until usage.

Starvation and analysis of morphogenesis by cell aggregation (conditioned medium) assay

A cell aggregation assay was performed as described in Vinet et al. (2014). Axenically growing DH1-10 *D. discoideum* cells (2 x 10^5 to 2 x 10^6 cells/ml) and/or *corA*$^-$ cells (2 x 10^5 cells/ml to 9.0 x 10^7 cells/ml) were centrifuged at 400*xg* for three minutes at 4°C, and then washed in the same PBM (0.02 M KH_2PO_4, 10 μM $CaCl_2$, and 1 mM $MgCl_2$, pH 6.2) buffer described above. The centrifugation and PBM wash steps were repeated two more times to free cells of bacteria, any factors that may promote cell aggregation, and remaining axenic HL5 nutrients. To induce starvation, the washed cell pellets were resuspended in PBM at a density of 1.0 x 10^7 cells. For analysis of cell aggregation responses, the starved cells were seeded into a 24-, and 48- well plates at a density of 20, 10, 5, and 2.5 x 10^4 cells/cm^2, and incubated in PBM for 30 to 60 minutes

to allow adhesion. The cells were incubated with PBM as negative control, and conditioned medium (CM) as positive control for between four to eight hours and overnight (16 h) at 22°C. After four to eight hours and overnight incubation at 22°C, images of cell aggregation were captured using a Carl Zeiss microscope. For the analysis of cell aggregation, CM activity after heating, treatments with proteinase K, and phospholipase D, ammonium sulfate fractionation, ultrafiltration, and chromatographic methods, the same cell aggregation assay described above was performed in this study.

Heat inactivation analysis of activity in the conditioned medium (CM)

Freshly thawed conditioned medium (CM) was separately heated at 60, 80, and 100 °C for 1 hour on a thermomixer (Eppendorf). For detection of cell aggregation activity in heated-CM, DH1-10 *D. discoideum* cells (2 x 10^5 cells/ml to 2.0 x 10^7 cells/ml) were seeded at a density of 20, 10, 5, and 2.5 x 10^4 cells/cm^2 on a 24-well plate, and incubated with PBM (0.02 M KH_2PO_4, 10 μM $CaCl_2$, and 1 mM $MgCl_2$, pH 6.2) as a negative control, untreated CM as a positive control, 60 °C, 80 °C, and 100°C heated-CM for between four and eight hours, and overnight (16 h) at 22 °C. After four to eight hours and overnight incubation at 22°C, aggregation of cells was monitored and photographed by using a Carl Zeiss microscope, as described above.

Proteinase K analysis of activity in the conditioned medium (CM)

To determine the protein nature of the conditioned medium (CM), each 1 ml of freshly thawed CM was treated for one hour at 37 °C with 5 μl heat-inactivated proteinase K (Roche), 5 μl proteinase K (20 mg/ml in PBM containing 0.02 M KH_2PO_4, 10 μM

$CaCl_2$, and 1 mM $MgCl_2$, pH 6,2) and 143 µl Roche protease inhibitor cocktail mixed with proteinase K (Roche). In addition, 1 ml of CM was incubated with 143 µl Roche protease inhibitor cocktail only. For proteolytic inactivation, proteinase K-treated CM was treated with Roche protease inhibitor cocktail. The treated-CM was then examined for cell aggregation activity in the DH1-10 *D. discoideum* strains (2×10^5 to 2.0×10^7 cells/ml) using the cell aggregation assay, as described above.

Phospholipase D analysis of activity in the conditioned medium (CM)

The freshly thawed conditioned medium (CM) sample was incubated with 1000 units/ml of phospholipase D for 1 hour at 37 °C [Wang, 1999]. The treated CM was assayed for cell aggregation activity in the wild-type DH1-10 *D. discoideum* strains (0.2×10^6 to 2×10^6 cells/ml), as described above.

Conditioned Medium (CM) purification by ammonium sulfate precipitation

Conditioned medium (CM) was treated with ammonium sulfate to reach 20%, 40%, 60%, and 80% saturation, and allowed to stir for 1 hour at 4 °C (the amount of ammonium sulfate required to attain the percentage of saturation was determined from the following website: http://www.encorbio.com/protocols/AM-SO4.htm). In order for factors to be saturated completely, each addition of 20%, 40%, 60%, and 80% ammonium sulfate to the CM solution was done at intervals of one hour. The precipitates were centrifuged at 10000*xg* for 15 minutes at 4 °C, and the resulting supernatant was discarded. The collected precipitates were suspended in PBM containing 0.02 M KH_2PO_4, 10 µM $CaCl_2$, and 1 mM $MgCl_2$ (pH 6.2). To remove the excess ammonium

sulfate, the precipitates were dialyzed overnight at 4°C in PBM using Molecular Porous Regenerated Cellulose Dialysis Membrane Tubings (Spectra/Por®). CM sample recovery was completed according to the Spectra/Por's and/or Thermo Scientific's instructions. Next, 20%, 40%, 60%, and 80% saturated precipitates were examined individually for cell aggregation activity in the DH1-10 *D. discoideum* cells (0.2 x 10^6 to 2 x 10^6 cells/ml) using the cell aggregation assay. In addition, as a comparison, other dialysis membrane products were utilized (Spectra/Por®: Float-A-Lyzer G2 devices, and Thermo Scientific: Slide-A-Lyzer Dialysis Cassettes).

Fractionation of starvation conditioned medium (CM) by ion exchange chromatography

An anionic HiTrap™ Q XL or a cationic HiTrap™ SP XL (GE Healthcare Life Sciences) column was washed with PBM (0.02 M KH_2PO_4, 10 μM $CaCl_2$, and 1 mM $MgCl_2$, pH 6.2), and then washed with 1 M NaCl in PBM buffer on an Äkta-FLCP system (GE Healthcare Life Sciences). Fifty ml freshly thawed conditioned medium (CM) solution was loaded on the columns. During this loading step, the flow-through fraction from the columns was collected. All fractionation steps were done at 4 °C. After increasing the salt concentration of the buffer to 1 M, attached molecules were eluted selectively from the columns with a gradient of 0 – 1 M NaCl for 10 and/or 20 min at a flow rate of 1 ml/min. Each 1 ml of fractions was collected manually in a 1.5-ml tube, and dialyzed overnight at 4 °C in PBM using Slide-A-Lyzer Dialysis Cassettes (10 K MWCO; Thermo Scientific). After dialysis, CM fraction sample recovery was completed following the Thermo Scientific's instructions. The pooled fractions were diluted at 1:1,

1:3, 1:7, or 1:15 with PBM, and then monitored individually for cell aggregation responses in both the wild-type DH1-10 *D. discoideum* strains (0.2×10^6 to 2×10^6 cells/ml) and coronin A-deficient cells (0.2×10^6 to 0.9×10^6 cells/ml) using the cell aggregation assay. Fractions that displayed aggregation activity were probed for protein detection by silver staining, characterized, and sequenced by mass spectrometry in collaboration with a mass spectrometry specialist at the Proteomics Core Facility in Biozentrum (Universität Basel), and frozen at (-80 °C) in liquid nitrogen until thawed. As an improvement for fractionation, we also used ultrafiltration membranes (Ultracel® 100 kDa Ultrafiltration Discs; Millipore) to concentrate active factors from the CM solution. Five hundred ml of freshly thawed CM was concentrated with ultrafiltration membranes to fifty ml, which was then loaded on the columns. Following column washing, the fractions were collected as previously described.

SDS-PAGE, coomassie blue, and silver staining

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular weight of active fractions eluted from ion exchange or size exclusion chromatography [Laemmli, 1970]. Running gels were prepared by using 30% acrylamide: 0.8% bisacrylamide (AppliChem), 2 M Tris/HCl (Sigma; pH 8.8), 20% SDS (Bio Rad), distilled water, 10% ammonium persulfate (APS; AppliChem), and tetramethylethylethylenediamine (TEMED; AppliChem). Stacking gels were prepared by mixing 30% acrylamide: 0.8% bisacrylamide (AppliChem), 0.5 M Tris/HCl (Sigma; pH 6.8), 20% SDS (Bio Rad), distilled water, 10% APS (AppliChem), and TEMED (AppliChem). SDS sample buffer (20% SDS, 100% glycerol (Fluka), 2 M Tris (Sigma;

pH 6.8), 50 mg/ml bromphenol blue (Merck), 3.855 mg/ml dithiothreitol (DTT; Sigma) was used to prepare conditioned medium fraction samples, which were heated for five minutes at 90 °C, and then loaded in gels. After running gels at 160 V for two hours, they were stained with coomassie blue (5 g/L coomassie brilliant blue R-250 (Sigma), 98% acetic acid (Synopahrm), 98% methanol (Schweizerhall)) or silver (60 ml acetone stock, 1.5 ml trichloroacetic acid (TCA) stock, 25 ul 37% HCHO, 100 ul $Na_2S_2O_3 \bullet 5H_2O$ in 60 ml water, 0,8 ml $AgNO_3$ in 0.6 ml 37% HCHO, and 60 ml water, 1.2 g Na_2CO_3 in 25 ul 37% HCHO, 25 ul $Na_2S_2O_3 \bullet 5H_2O$, and 60 ml water, 1% glacial acetic acid, distilled water). PageRuler Plus Prestained Protein Ladder (Thermo Scientific) was used to estimate the molecular weight of active fractions.

Further fractionation of conditioned medium (CM) fractions from ion exchange chromatography by size exclusion chromatography

As described above, the eluted fractions were thawed from -80 °C, and then subjected to size exclusion chromatography on the Superdex-200 column (GE Healthcare Life Sciences). Initially, the Superdex-200 column was equilibrated with distilled water (filtered and degassed) for one hour at a flow rate of 0.5 ml/min. The column was then equilibrated with PBM (0.02 M KH_2PO_4, 10 μM $CaCl_2$, and 1 mM $MgCl_2$, pH 6.2) again for one hour at a flow rate of 0.5 ml/min. Prior to loading fractions on the column, active eluted fractions were combined, and then concentrated with 100,000 NMWL (Nominal Molecular Weight cut-off Limit) Amicon® Ultra-4 centrifugal filter devices (Millipore) to a final volume that was diluted with PBM to 500 uL. Prior to loading on Superdex-200, diluted factors were clarified by microcentrifugation for 10 minutes at 13,000*xg*. Then,

this was loaded on the column, and resulting fractions were tested for cell aggregation responses in the wild-type DH1-10 *D. discoideum* strains (0.2×10^6 to 2×10^6 cells/ml) and coronin A-deficient cells (0.2×10^6 to 0.9×10^6 cells/ml), as described above. As with fractionation of conditioned medium (CM) by ion exchange chromatography, the fractions with cell activities were subjected to silver staining, and mass spectrometry for protein detection, characterization, and sequencing.

4. RESULTS

Cell aggregation activity of the starvation conditioned medium (CM) by heat and proteinase K sensitivity analyses

Recent work has provided an insight into coronin A-induced development in *Dictyostelium discoideum* [Vinet et al., 2014]. Incubation of *D. discoideum* cells with conditioned medium has shown that coronin A initiates the cAMP relay resulting in cell aggregation [Vinet et al., 2014]. In *D. discoideum* with deletion of coronin A, the failure of initiating morphogenesis following incubation with conditioned medium (CM) suggests that induction of development is coronin A dependent [Vinet et al., 2014]. Depletion of food causes cells to starve, resulting in non-proliferating cells that secrete developmental factors essential for the initiation of early development [Bonner, 1952; Lam & Siu, 1981; Gomer et al., 1991; Burdine & Clarke, 1995; Iijima et al, 1995; Brown & Firtel, 1999; Mahadeo & Parent, 2006; Gomer et al., 2011]. Therefore, our original aim in this study was to determine whether a cell aggregation-inducing substance was heat-stable, and the developmental activity that occurred in the transition from starvation to cell aggregation of *D. discoideum* was due to a protein(s) present in the conditioned medium (CM) harvested from starved cells, as described in Material and Methods. Cell aggregation activities in the wild-type *D. discoideum* DH1-10 strains were investigated during heat treatment.

First, thawed conditioned medium (CM) was separately heat-treated for 1 hour at 60, 80, and 100 °C. It was shown that the cell aggregation activity of heated-CM significantly decreased at 60, 80, and 100 °C, as indicated in Fig. 5 (K, L, O, P, S, and T). In previous studies, the CM activity was also examined at 35, 80, and 100 °C by Iijima et

al. (1995). However, in our case, CM in this analysis was prepared from cells that were starved for eight hours, whereas Iijima et al. (1995) starved cells for 16 hours prior to preparing the starvation medium. Our results suggest that a CM factor(s) proves sensitive to heat treatment at all the incubation temperatures. Furthermore, in the presence of free-nutrient buffer (PBM), we observed spontaneous cell aggregation that appeared to be dependent on specific cell densities (Fig. 5. A, and B; see also Fig. 6. A, and B), which is consistent with the observations by Hashimoto et al. (1975), Gomer et al. (2011), and a recent study by Nagano and Sakurai (2013) demonstrating that clusters of starving *D. discoideum* cells play a key role in the cAMP relay during multicellurarity [Siegert & Weijer, 1995; Kriebel & Parent, 2004].

To further characterize the molecular nature of an active factor(s), conditioned medium (CM) was subjected to the proteinase K treatment [Morihara & Tsuzuki, 1975; Han et al., 2004; Radisky et al., 2006]. The induction for cell aggregation was significantly reduced or abolished after the treatment with proteinase K (Fig. 6. N, O, and P), suggesting that the activity of a component(s) present in CM is lost after proteinase K treatment. As a matter of fact, when CM was exposed to both heat treatment and proteolysis, active CM components in the treated sample lost their cell aggregation activities significantly.

Our heat and proteinase K analyses suggest that it is most likely that the early development-inducing substance(s) from the starvation conditioned medium (CM) may be both a heat-, and protease-sensitive protein(s), which appears to be responsible for causing cells to initiate their development in response to the nutritional deprivation. Consistent with the above-mentioned protease-sensitivity of the CM proteins, we

demonstrated that Roche protease inhibitor cocktail was able on its own to impact the early development of *D. discoideum*, indicating the possibility that a proteolytic cleavage may be essential for the full cell aggregation function in the starvation state (Fig. 6 S) [Rossomando et al., 1978].

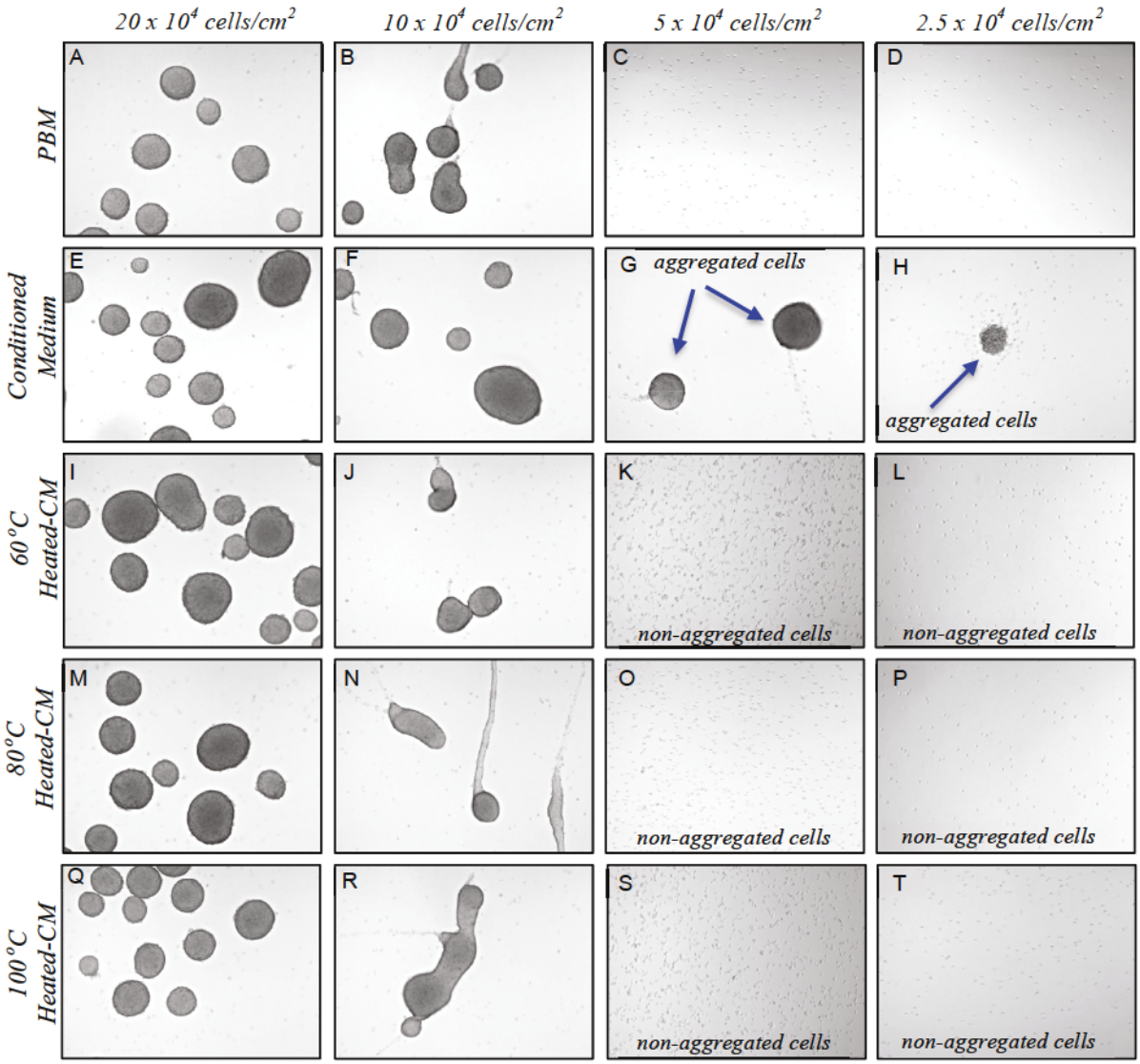


Fig. 5. Cell aggregation assay after heat treatment, and effect of heat on conditioned medium (CM) during *D. discoideum* development. Wild-type DH1-10 cells at different cell densities were incubated with CM heated at 60, 80, and 100 °C. After 16 h, cells at the high densities aggregated in the presence of heated CM (I, J, M, N, Q, and R) and absence of CM (negative control; PBM). Cell aggregation is defined as a group of cells that aggregate together to form a multicellular structure.

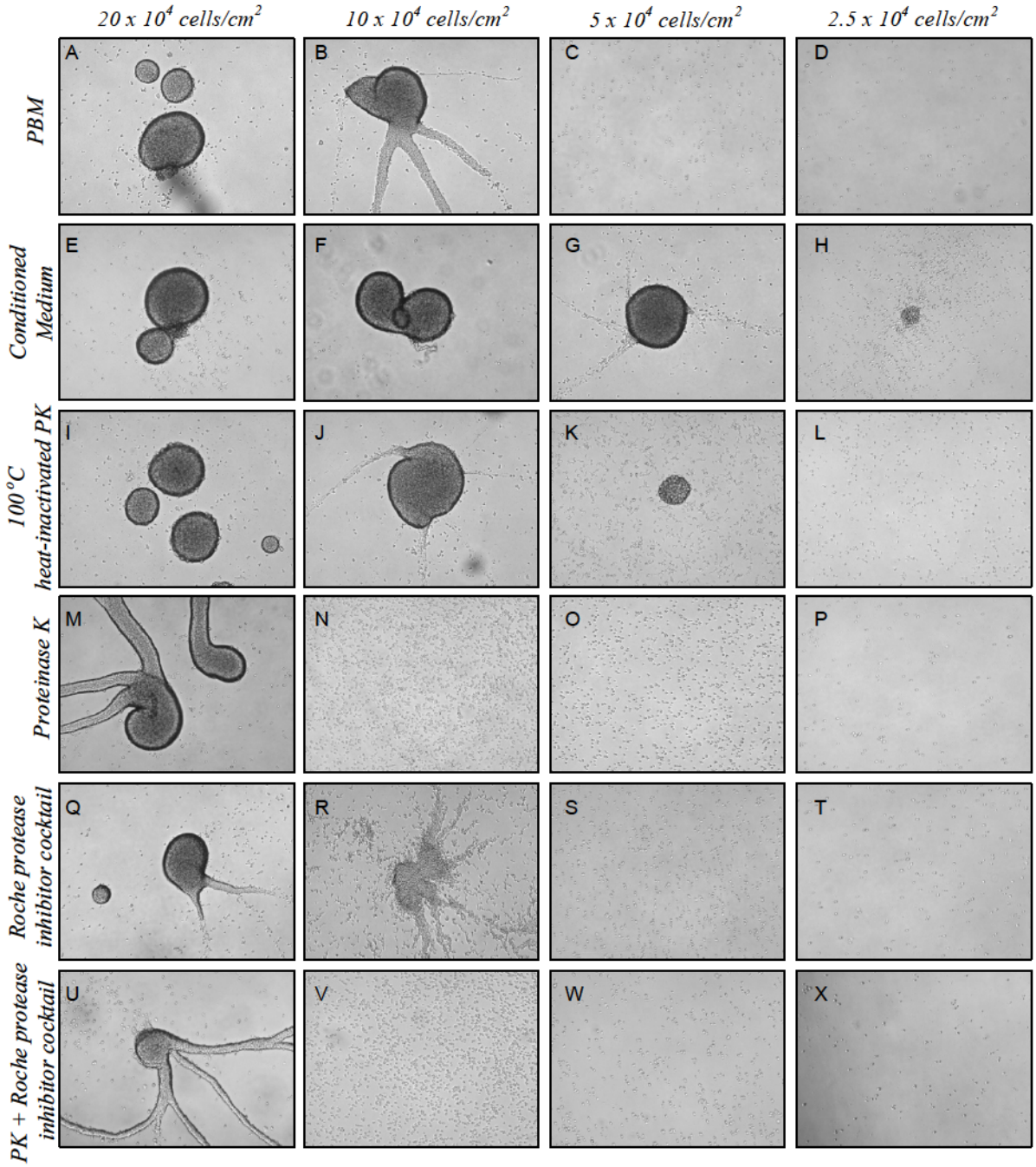


Fig. 6. Cell aggregation after proteinase K treatment, and effect of proteinase K on conditioned medium (CM) during *D. discoideum* development. Each 1-ml CM sample was treated with heat-inactivated proteinase K at 100 °C for 1 hour, proteinase K alone at 37 °C for 1 hour, Roche protease inhibitor cocktail alone, or a combination of both proteinase K and Roche protease inhibitor cocktail at 37 °C for 1 hour. Wild-type DH1-10 cells were incubated with proteinase K-treated CM for 16 hours. As with the results for heat sensitivity, cells at the high densities aggregated in the absence of CM (A, and B).

Analysis of activity on the conditioned medium (CM) by phospholipase D

Based on the recent findings revealing that phosphatidylcholine lipids are present in an abundant quantity from the aggregating *D. discoideum* cells [Fernandez-Lima et al., 2014], and to determine whether these lipids secreted by aggregating cells in response to the starvation for 16 hours have a role in the initiation of development, we tested the effect of phospholipase D, which acts specifically on phosphatidylcholine molecules [Zouwail et al., 2005; Ray et al., 2010; Enzo Life Sciences, 2014], on cell aggregation. We incubated the conditioned medium (CM) sample with phospholipase D for 1 hour at

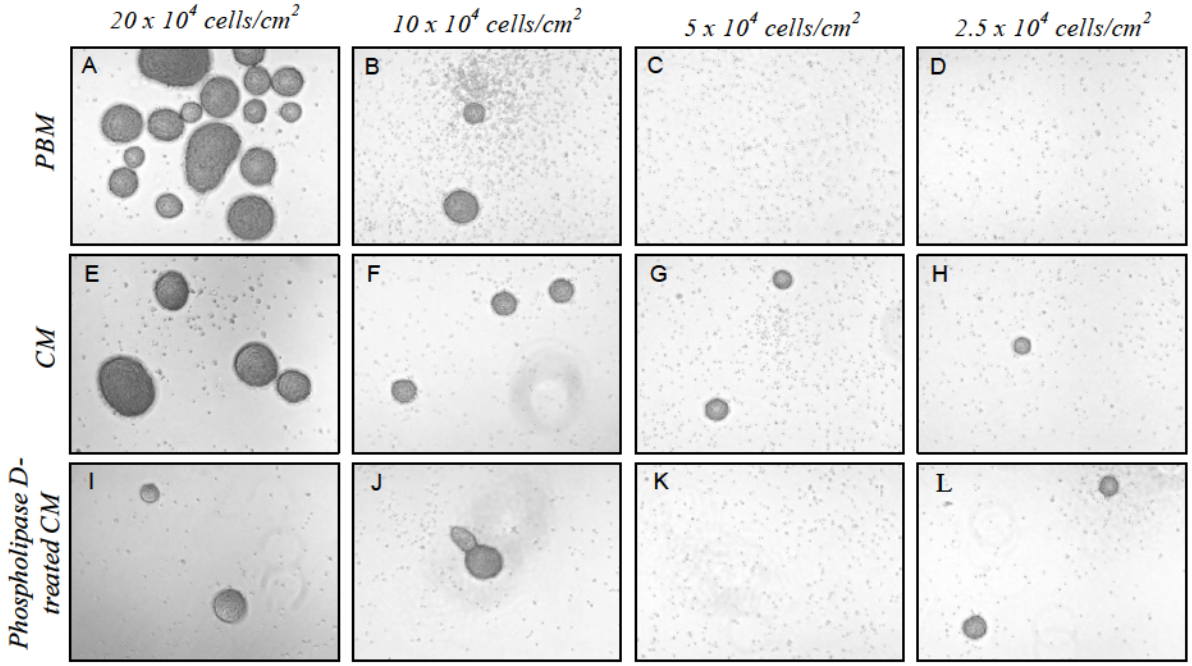


Fig. 7. Cell aggregation assay after phospholipase D treatment and effect of phospholipase D on conditioned medium (CM) during *D. discoideum* development. Wild-type DH1-10 cells at different cell densities were incubated overnight (16 h) with PBM (negative control), CM (positive control), and phospholipase D-treated CM.

37° C. Next, the treated CM was assayed for cell aggregation activity in the wild-type DH1-10 *D. discoideum* strains, as described previously. We observed that the CM activity was variable ranging from no cell aggregation activity at the cell density of 5 x

10^4 cells/cm^2 to cell aggregation activity at the density of 2.5 x 10^4 cells/cm^2 (Fig. 7. K, L). As an additional criterion, we tested again the effect of phospholipase D on the CM sample. Again, a variable activity was observed (Fig. 8), suggesting that the activity of phosphatidylcholine is not all-or-none but may be involved in contributing to the aggregation process [Ray et al., 2011].

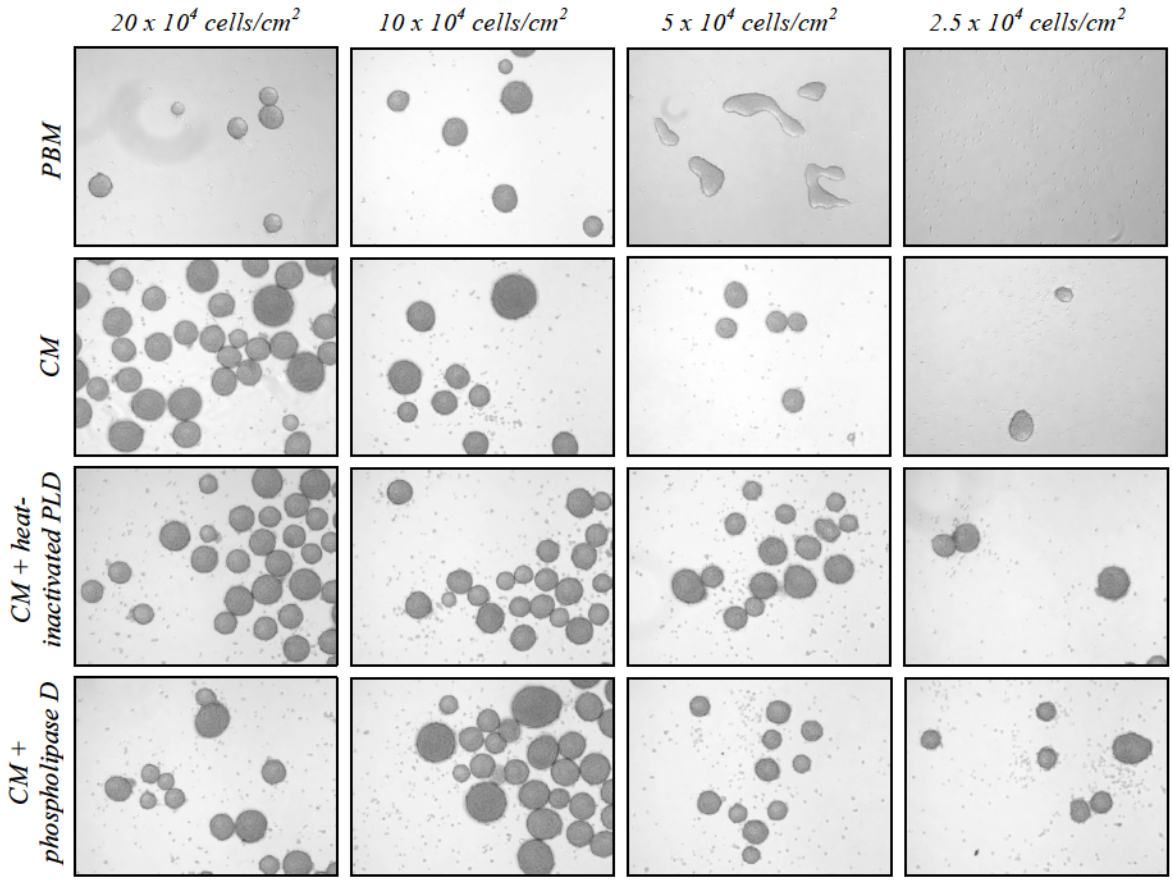


Fig. 8. Cell aggregation assay after phospholipase D treatment and effect of phospholipase D on conditioned medium (CM) during *D. discoideum* development. Wild-type DH1-10 cells at different cell densities were incubated overnight (16 h) with PBM (negative control), CM (positive control), CM + heat-inactivated phospholipase D, and phospholipase D-treated CM.

Conditioned medium (CM) purification by ammonium sulfate precipitation

To examine the effect of an active component(s) on the developmental capacity of *D. discoideum*, freshly harvested conditioned medium (CM) was purified with 20%, 40%, 60%, and 80% ammonium sulfate. We observed that partial aggregation activity was at the intervals of between 20 – 80%. Low cell aggregation activity was detectable in the cells at the high densities (Fig. 9. I, M, Q, U, and V). These results suggest that in the CM precipitates the abundance and homogeneity of active components were not significantly increased at the ammonium sulfate intervals of 60 – 80%, whereas very low activity was seen in 20 – 60%. In addition, we tested a variety of commercially available dialysis membranes (Spectra/Por®: Float-A-Lyzer G2 devices, Molecularporous Regenerated Cellulose Dialysis Membrane Tubings, and Slide-A-Lyzer Dialysis Cassettes) to purify the active factors(s) from the medium sample containing ammonium sulfate, however we were not able to recover cell aggregation activity in most CM precipitates. Apparently, ammonium sulfate precipitation reduced or inhibited the activity significantly. As a result, this attempt was not successful, and this purification strategy was not further pursued.

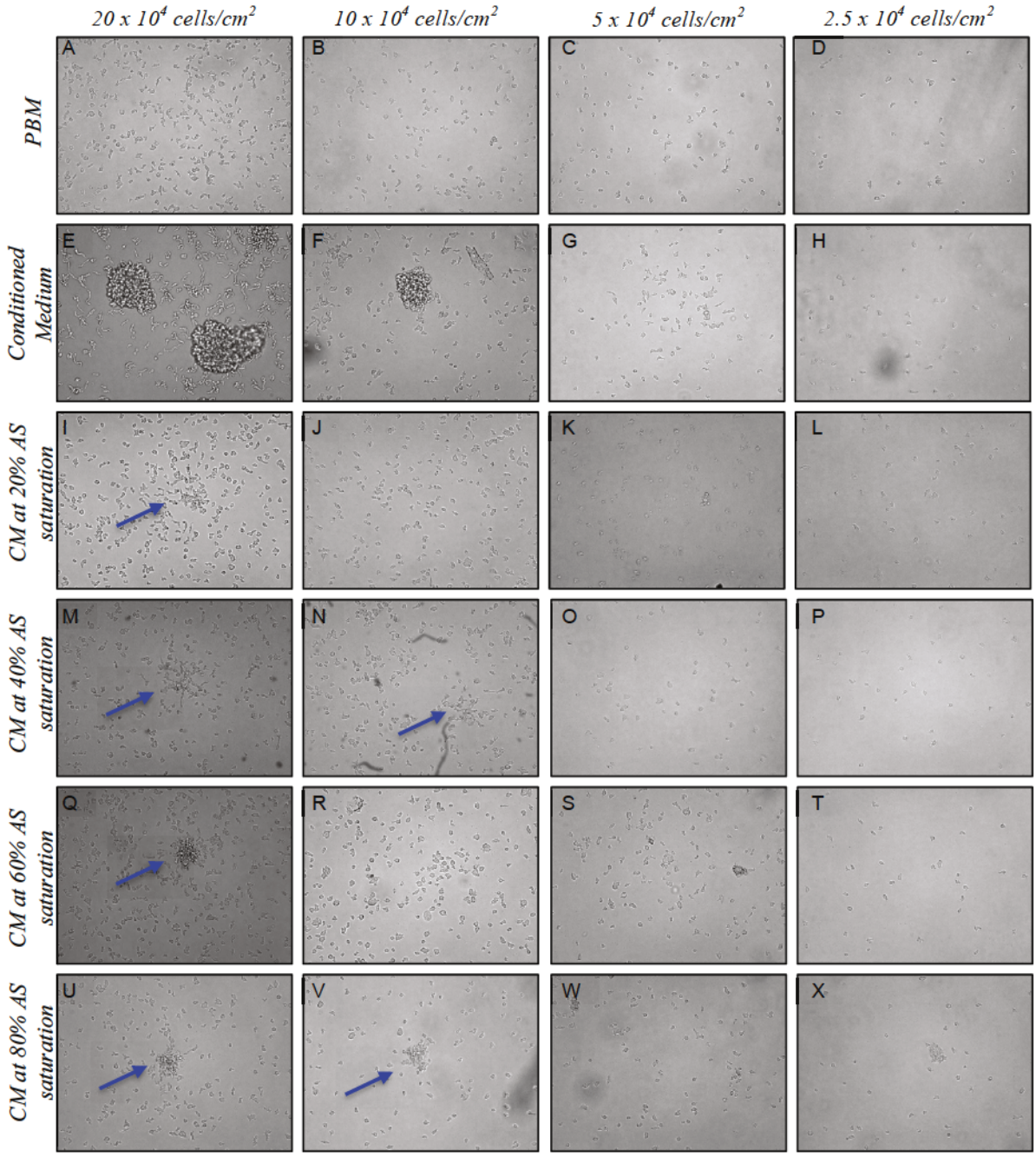


Fig. 9. Cell aggregation assay after purification of an active substance(s) from conditioned medium (CM) by ammonium sulfate (AS) precipitation. After 16 h of aggregation assay, low cell aggregation activity was detectable in the precipitates at the ammonium sulfate intervals of 20 – 80% (as indicated by blue arrows).

Characterization and concentration of the secreted factors in the conditioned medium sample

In the ammonium sulfate precipitation experiments, although a purified active factor(s) had an observed low aggregation-inducing activity due to the co-precipitation with unknown components [Marshak et al., 1996] or permanent denaturation of substances by ammonium sulfate, it caused some limited cell aggregation. Next, we wanted to examine the developmental effect of starvation conditioned medium (CM) at different concentrations on *D. discoideum* DH1-10 strains, and determine in more detail which molecular weight of secreted factors exerted a developmental effect on starving cells. We observed that after 16 hour of incubation, induction of development occurred in cells from high to low cell densities: 20, 10, 5, and 2.5 x 10^4 cells/cm2 in a 24-well plate (Fig. 10). Conditioned medium concentrated with the Millipore 3,000 NMWL (Nominal Molecular Weight cut-off Limit), 10,000 NMWL, 50,000 NMWL, or 100,000 NMWL ultrafiltration membranes exhibited similar capabilities for promoting cell aggregation. These results demonstrate that CM concentrates (active secreted factors) from 3,000 to 100,000 NMWL contained the developmental activity. Apparently, the active factors in conditioned medium have a higher molecular weight above 100 kDa. Moreover, changing the assay format from a 24 to 48 well plate in order to reduce the dose required for the cell aggregation examination resulted in similar activities (data not shown).

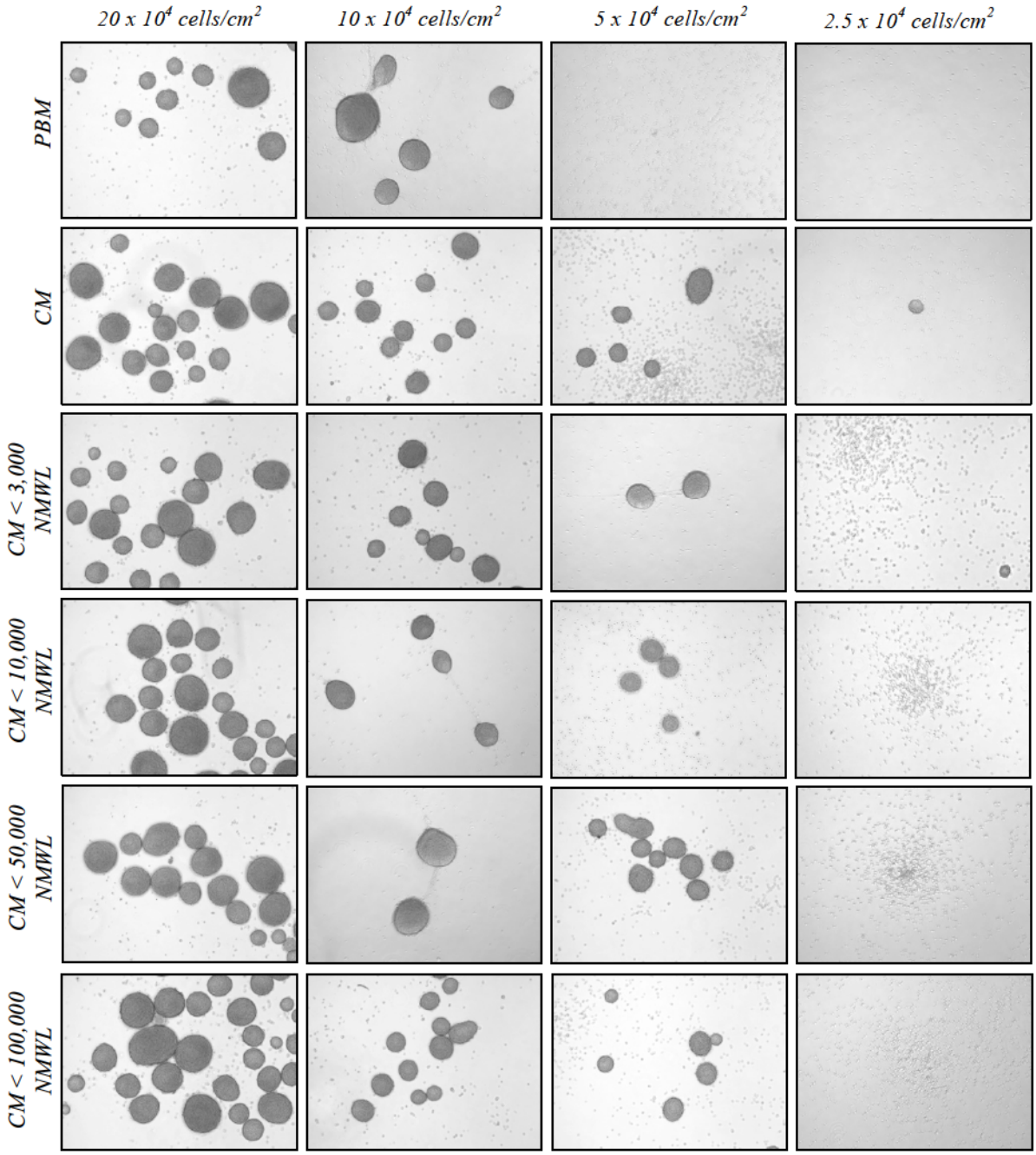


Fig. 10. Concentration of conditioned medium (CM) by ultrafiltration membranes (Millipore). After 16 h of aggregation assay, CM factors with molecular weight of 3,000 to 100,000 Dalton induced morphogenesis in *D. discoideum* DH1-10 strains settled on 24 and 48 well plates.

Purification of conditioned medium by ion exchange chromatography

Based on the above results, where the conditioned medium (CM) factors can be concentrated by ammonium sulfate and ultrafiltration membranes, and are sensitive to proteolysis, they appeared to be proteins. Furthermore, the 100 kDa Amicon ultrafiltration provides a first purification step by eliminating proteins and/or protein complexes with a molecular weight below 100 kDa from the active conditioned medium (CM). To narrow down the number of CM components that would be potential candidates for inducing cell aggregation in a coronin A-dependent manner, we used ion exchange chromatography to fractionate the freshly harvested CM sample into 20 fractions, 10 of which were eluted from an anionic HiTrap™ Q XL column and the other 10 fractions from a cationic HiTrap™ SP XL (GE Healthcare Life Sciences) with a linear gradient of 1 M NaCl buffer for 10 min at a flow rate of 1 ml/min. We observed that there were no peaks of CM activity in all the fractions eluted from the HiTrap™ SP XL column, whereas in the fractions eluted from the HiTrap™ Q XL column we found two peaks of CM activity (Fig. 11). When both the Q and SP fractions were assayed individually for cell aggregation responses in the DH1-10 *D. discoideum* strains, we observed most aggregation activities within the fractions Q6 to Q10, however no activities were observed in any of the SP fractions (data not shown). Thus, we conclude that no cell aggregation was induced by purified fractions from the cationic HiTrap™ SP XL column.

Our preliminary studies demonstrate that the mutants lacking coronin A failed to form aggregates in the presence of conditioned medium (CM) [Vinet et al., 2014]. To determine whether this failure was due to the lack of sensing of a secreted factor by

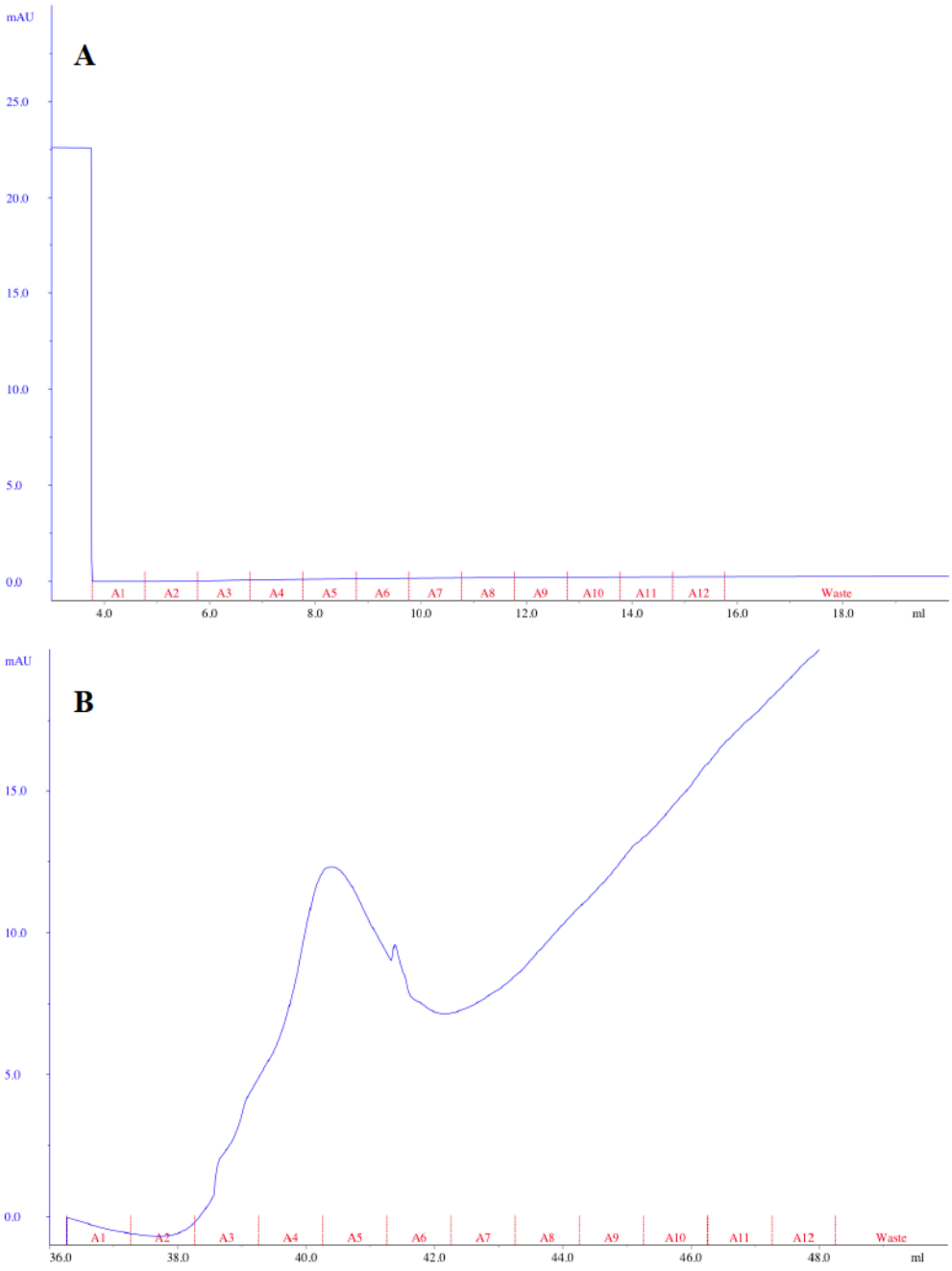


Fig. 11. Cationic HiTrap™ SP XL and anionic HiTrap™ Q XL chromatography of concentrated conditioned medium (CM) factors. Active fractions were eluted with a linear gradient of NaCl from 0 to 1 M. **A.** No peak activity fractions from the HiTrap™ SP XL column. **B.** Two peak activity fractions from the HiTrap™ Q XL column. Fractions with aggregation-inducing properties eluted at approximately 0,5 M NaCl. Blue line is the milliabsorbance units at A280 nM (mAU); fractions designated as A1 to A10 in red (fraction numbers).

coronin A, factors purified from the anionic HiTrap™ Q XL column were tested for cell aggregation activity on wild-type cells and *corA*⁻ cells. We found that when the starved *corA*⁻ mutants were treated with the conditioned medium (positive control) they failed to aggregate, and formed few aggregates when incubated with the anionic fractions obtained from ion exchange chromatography, as compared with the coronin A-expressing cells (Fig. 12). These findings suggest that coronin A is essential for cell aggregation induced by the active fractions obtained from ion exchange chromatography during the starvation response. Furthermore, we attempted to improve factor separation by changing the gradient from a 10-min to 20-min interval with the same 1 M NaCl buffer, and ion exchange chromatography columns collecting 20 fractions sequentially from both HiTrap™ Q XL and HiTrap™ SP XL columns. When we incubated the wild-type and coronin A-deficient cells with 20 Q fractions, we observed the presence of the cell aggregation activities in the wild-type cells, but not in the *corA*⁻ mutants (data not shown). However, no marked improvement in fractionating the conditioned medium sample was observed.

By comparing the difference in phenotypes between the wild-type cells and *corA*⁻ mutants throughout the starvation-induced cell aggregation stage, we confirmed that factors present in the Q fractions were strongly dependent on coronin A. Since these active Q fractions were considered to be candidates for involvement in coronin A-induced cell aggregation, we further characterized them by SDS-PAGE and silver staining, and then sent them to a mass spectrometry specialist at the Proteomics Core Facility in Biozentrum for characterization and sequencing analyses.

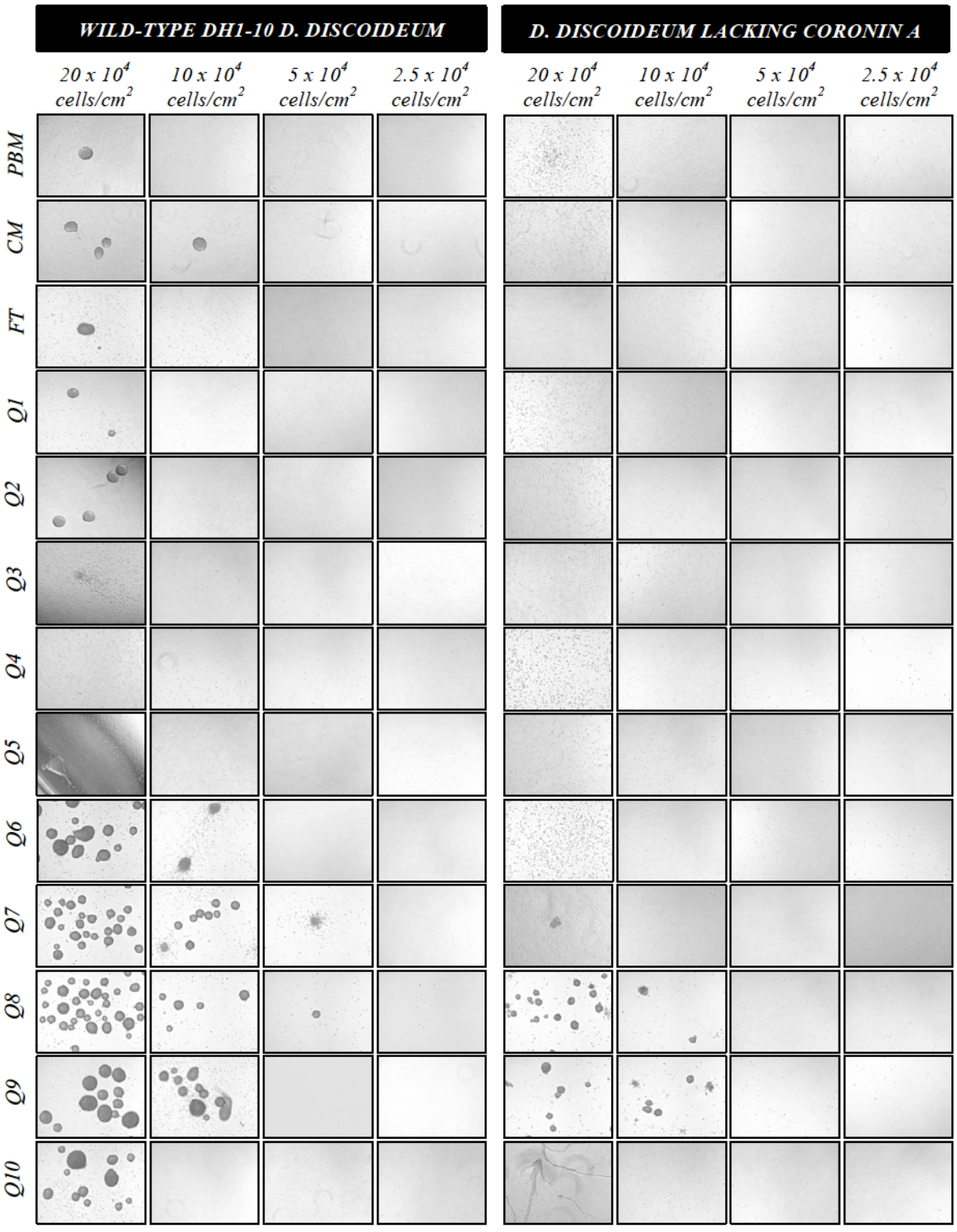


Fig. 12. Cell aggregation in the wild-type DH1-10 strains and coronin A-deficient strains of *D. discoideum*. Both the wild-type and coronin A-deficient cells at the densities of 20, 10, 5, and 2.5 x 10^4 cells/cm^2 were starved in PBM, and then incubated with PBM (negative control), conditioned medium (CM; positive control), flow-through (FT), and chromatographic elutes of the anionic column (Q1 to Q10). Cell aggregation assays were done overnight (16h).

The purity of studied factors probed by silver staining revealed different secreted factor bands visible in the range from 25,000 to 130,000 Dalton (Fig. 13). As indicated in Fig. 13, different factors present in the silver stained-gel were secreted by starving *D. discoideum*. Moreover, our mass spectrometry (MS) analyses resulted in a large number of sequenced proteins from each active fraction (Table 1). Using these MS analyses, we proceeded to identify potentially interesting MS hits from by comparing them to studies published in dictyBase (www.dictybase.org), which revealed that some of the interesting hits within Table 1 belong to a group of secreted factors that mainly contribute to the early development process in *D. discoideum*, such as contact site A protein (CsaA, also known as GP80; 54 kDa), the countin-1 (CTNA; 26 kDa), counting factor 60 (CF60; 46 kDa), and conditioned medium factor (CMF; 80 kDa) [Harloff et al., 1989; Yoshida et al., 1997; Gomer et al., 1991; Brock & Gomer, 1999; Maeda & Kuwayana, 2000; Loomis, 2014].

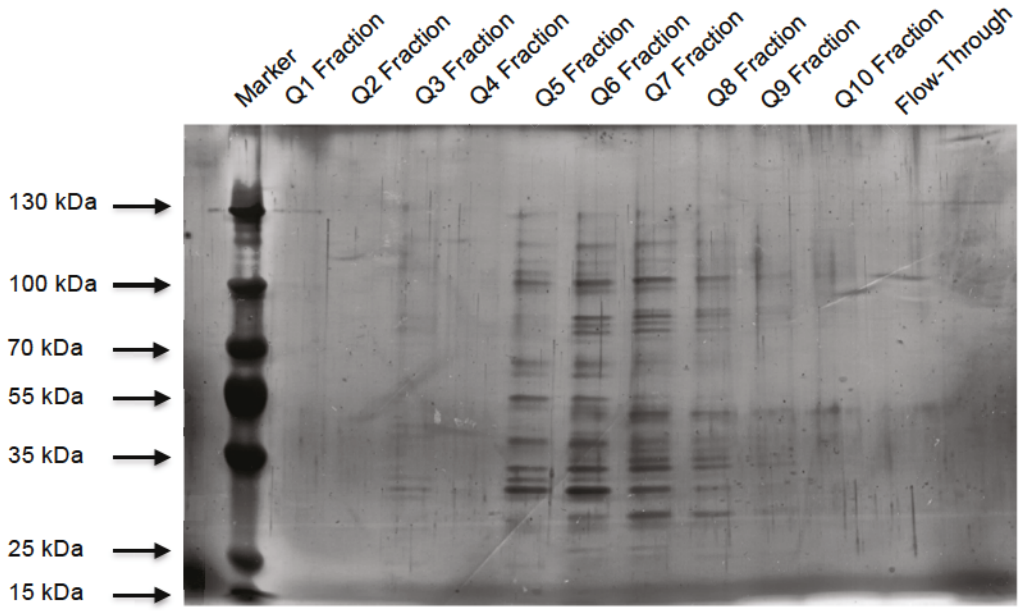


Fig. 13. SDS-PAGE and silver staining of active factors eluted from the anionic HiTrap™ Q XL column by ion exchange chromatography. Molecular weight of the proteins with cell aggregation activity are revealed at center in the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel.

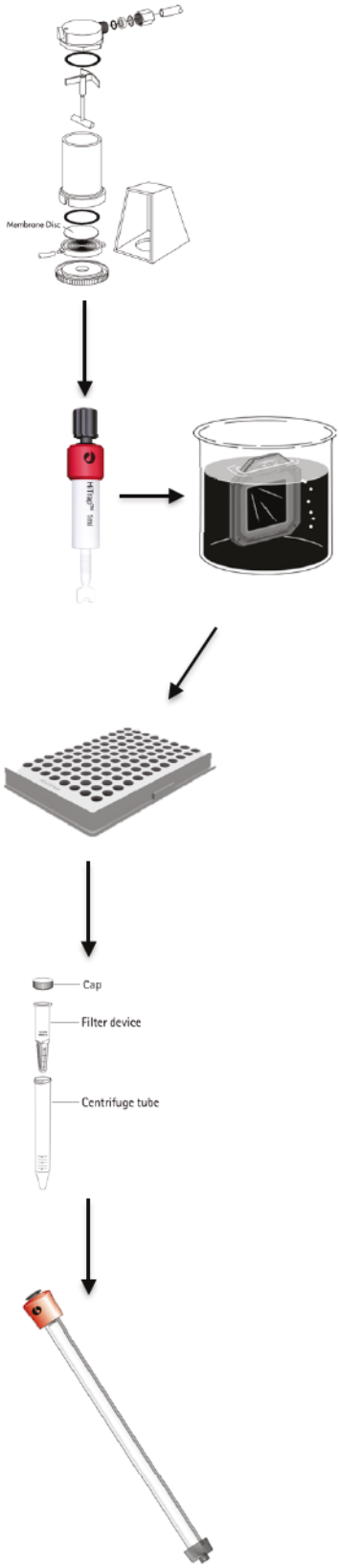
Table 1. The secreted factors identified by mass spectrometry after ion exchange chromatography

Proteins Sequenced by MS	Number of Proteins Identified by MS	Interesting Hits (score)
Conditioned Medium (positive control)	264	- Differentiation associated protein 2 (224) - Contact site A protein (75) - Countin-1 (73) - Counting factor 60 (70) - Conditioned medium factor (38)
Flow-Through	120	- Contact site A protein (64) - Countin-1 (58)
Q6 Fraction	91	- Contact site A protein (83) - Countin-1 (59) - Conditioned medium factor (57)
Q7 Fraction	65	- Contact site A protein (8) - Countin-1 (5) - Counting factor 60 (3) - Conditioned medium factor (3)
Q8 Fraction	68	- Countin-1 (8) - Counting factor 60 (7)
Q9 Fraction	53	- Countin-1 (8) - Contact site A protein (3)

Purification of peak activity fractions from ion exchange chromatography by size exclusion chromatography

On the basis of the mass spectrometry analyses, the number of identified proteins in the active fractions of anion-exchange chromatography is too large to further characterize by molecular biological techniques. As a consequence, further purification steps using size exclusion were established to improve the purity of the secreted factors in the conditioned medium (CM).

Using size exclusion chromatography, experiments were performed to narrow down to the conditioned medium factor as a possible candidate for cell aggregation dependent on coronin A. Similar to the chromatography steps detailed previously, we used the same columns (anionic HiTrap™ Q XL and cationic HiTrap™ SP XL from GE Healthcare Life Sciences), but with the exception that we concentrated a large volume (500 ml) of the conditioned medium solution through ultrafiltration membrane discs (Ultracel® 100 kDa Ultrafiltration Discs; Millipore) prior to loading on ion exchange chromatography columns. Thus, our purification procedure consisted of: 1) concentration of the conditioned medium through ultrafiltration; 2) fractionation of concentrated factors by ion exchange chromatography on the anionic HiTrap™ Q XL and cationic HiTrap™ SP XL columns; 3) conditioned medium activity assays on strains of DH1-10 *D. discoideum*; 4) additional concentration of active fractions by 100,000 NMWL Amicon® Ultra-4 centrifugal filter devices; 5) further separation of concentrated fractions on the Superdex-200 column (GE Healthcare Life Sciences); 6) and, finally, cell aggregation assays using elutes from size exclusion chromatography on both wild-type cells, and *corA*$^{-}$ mutants (Fig. 14).



1. Concentration of conditioned medium by Ultracel® 100 kDa Ultrafiltration Discs (Millipore)

2. Fractionation of concentrates (secreted factors) by ion exchange chromatography into fractions, dialysis of sample, and sample recovery

3. Conditioned medium assays in 48 well plates and observing cell aggregation responses under a microscope

4. Additional concentration of active fractions by 100,000 NMWL Amicon® Ultra-4 centrifugal filter devices

5. Further separation of concentrated fractions by size exclusion chromatography, and cell aggregation activity assays

Fig. 14. A workflow of secreted factor purification and characterization

Unlike cationic HiTrap™ SP XL, when concentrated conditioned medium was loaded on anionic HiTrap™ Q XL, the sample separated into several fractions of A7 to A10, as shown in Fig. 15. We speculated that the cell aggregation activity may be present in positively charged factors, which bound to the anionic column at pH 6.2. Thus, to examine whether the activity resided in fractions A7 to A10, we performed conditioned medium activity assays by incubating them, including non-activity peak fractions, with wild-type DH1-10 *D. discoideum* cells in a 48 well plate overnight. As shown in Fig. 16, the active fractions A7 – A10 (designated as Q7 – Q10) induced cell aggregation, whereas the other purified fractions A1 – A6 did not. Since the fractions tested on cells revealed cell aggregation inducing properties, and could be fractionated from the column with a linear NaCl gradient around 0.5 M, they were subjected to size exclusion chromatography on Superdex-200 column in an attempt to improve purification of the fractions from residual impurities for protein candidate identification. In addition, this enabled us to further assess whether the fraction(s) played a role in inducing cell aggregation. Before size exclusion chromatography, the active fractions were combined together, and then concentrated through 100,000 NMWL Amicon® Ultra-4 centrifugal filter devices. It was observed that cell aggregation factors eluted into three peaks after seven ml, as indicated in Fig. 17.

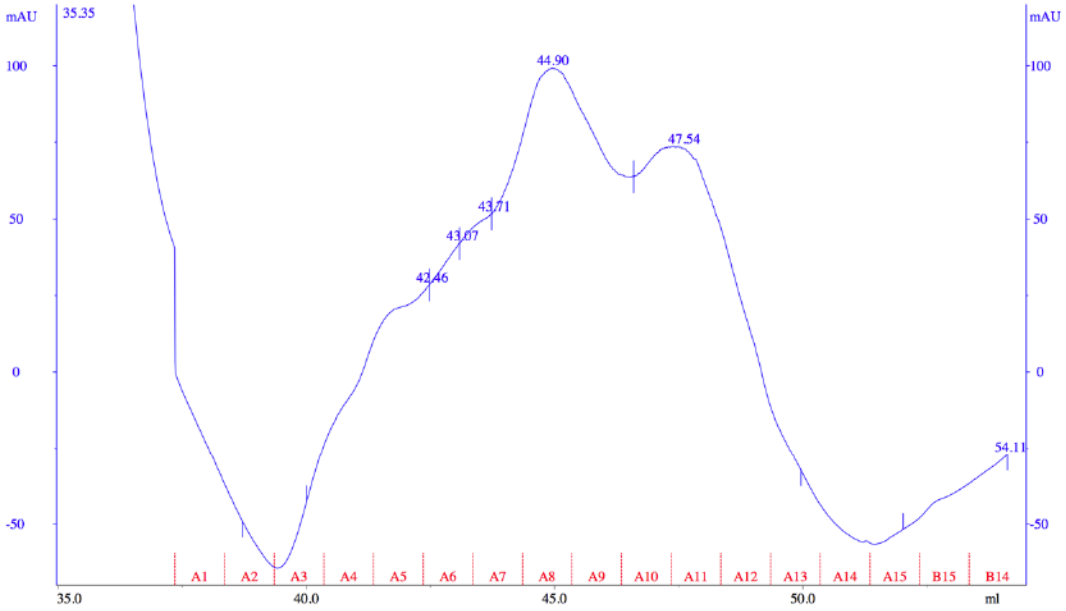


Fig. 15. Anionic HiTrap™ Q XL chromatography of concentrated conditioned medium (CM) factors. After concentrating CM with ultrafiltration membranes, this was loaded on the column. Active fractions were eluted with a linear gradient of NaCl from 0 to 1 M. Fractions, A7 – A10, with cell aggregation-inducing properties eluted at approximately 0,5 M NaCl. Blue line is the milliabsorbance units at A280 nM (mAU); fractions designated as A1 to A10 in red (fraction numbers).

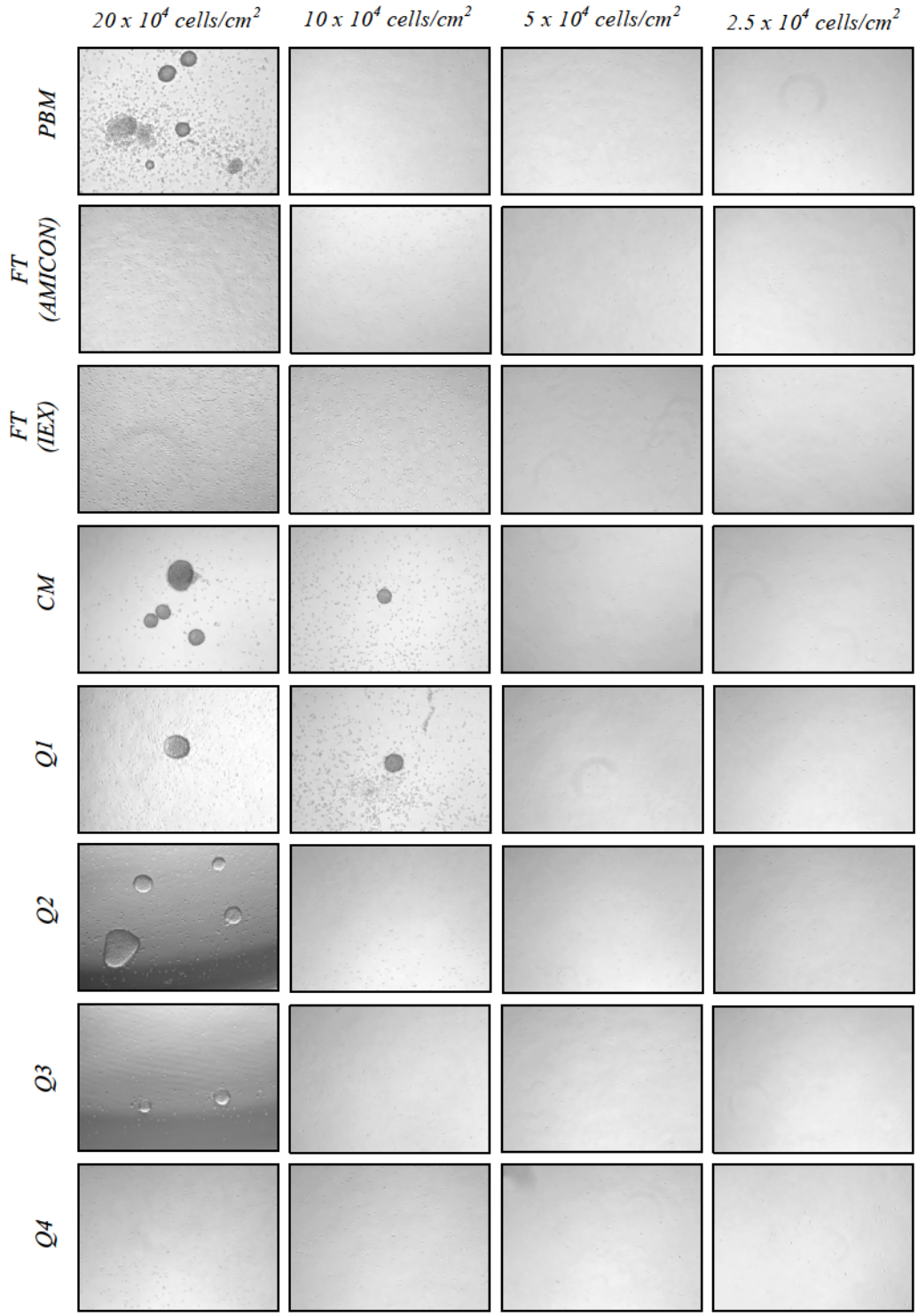


Fig. 16. Cell aggregation in the wild-type DH1-10 strains. Wild-type cells at the densities of 20, 10, 5, and 2.5 x 10^4 cells/cm^2 were starved in PBM, and then incubated with PBM (negative control), conditioned medium (CM; positive control), flow-through (FT), and chromatographic elutes (Q1 to Q10). Cell aggregation assays were done overnight (16h). *This figure continues on the next page.*

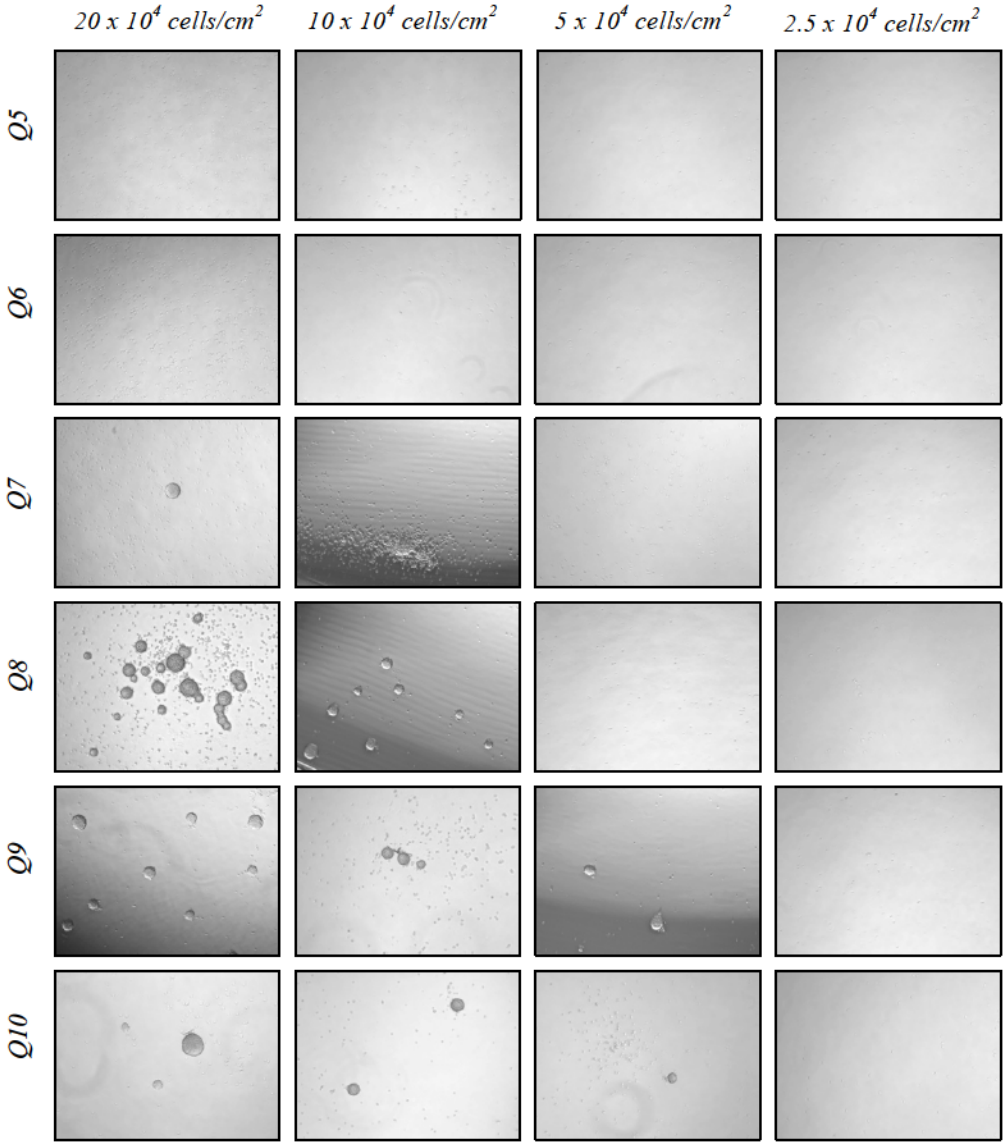


Fig. 16. (*continued*) Cell aggregation in the wild-type DH1-10 strains. Wild-type cells at the densities of 20, 10, 5, and 2.5 x 10^4 cells/cm^2 were starved in PBM, and then incubated with PBM (negative control), conditioned medium (CM; positive control), flow-through (FT), and chromatographic elutes (Q1 to Q10). Cell

By examining all 23 fractions for cell aggregation activities in wild-type and coronin A-deficient cells, it was observed that strains DH1-10 *D. discoideum* aggregated in response to most high peak fractions (Fig. 17). Whereas wild-type cells responded to developmental signals from active fractions SEC 12 to 16, almost all *corA*$^-$ mutants lacked detectable cell aggregation activity, thus not developing (Fig. 18; see the SEC 12 to SEC 16 fractions). *corA*$^-$ mutants, once treated with the fractions, ultimately remained at the pre-cell aggregation stage after 16 hours of incubation. Even when few mutants entered the stage, eventually they appeared to fail to aggregate completely. Apparently, these results suggest that the activation of coronin A is dependent on a specific-secreted factor(s) that persists in our fraction samples throughout the cell aggregation stage. Indeed, our findings from both ion exchange and size exclusion chromatography point towards active fractions as main regulators of the coronin A-mediated cAMP relay.

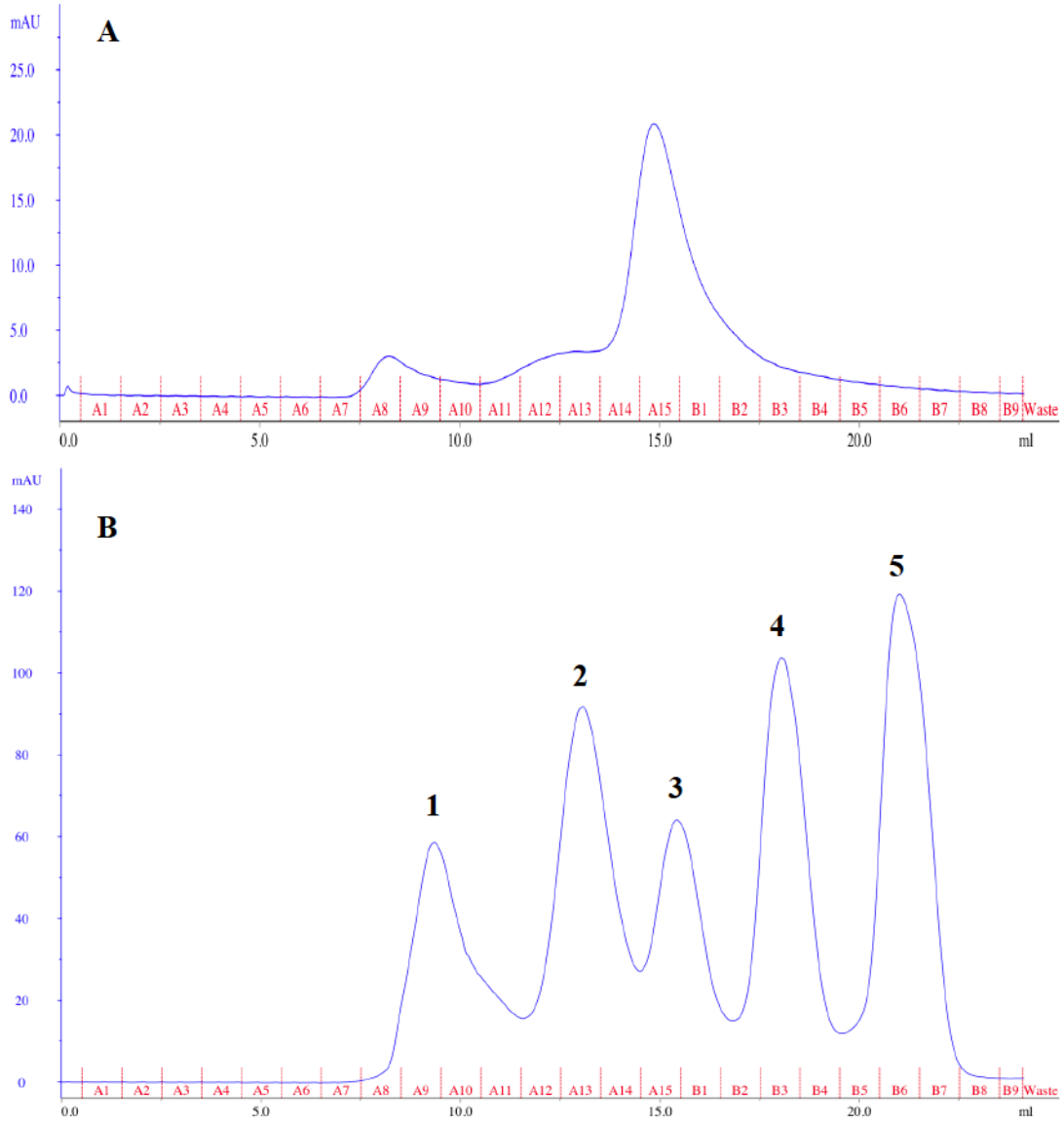


Fig. 17. Further fractionation of active fractions by size exclusion chromatography. **A.** Fractions with cell aggregation-inducing properties eluted after seven ml. Blue line is the milliabsorbance units at A280 nM (mAU); fractions designated as A1 to A15, and B1 to B9, in red, correspond to SEC fraction number of each sample (SEC 1 to SEC 23). **B.** Gel filtration marker standard. Peak 1, 2, 3, 4, and 5 correspond to thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1,350 Da)

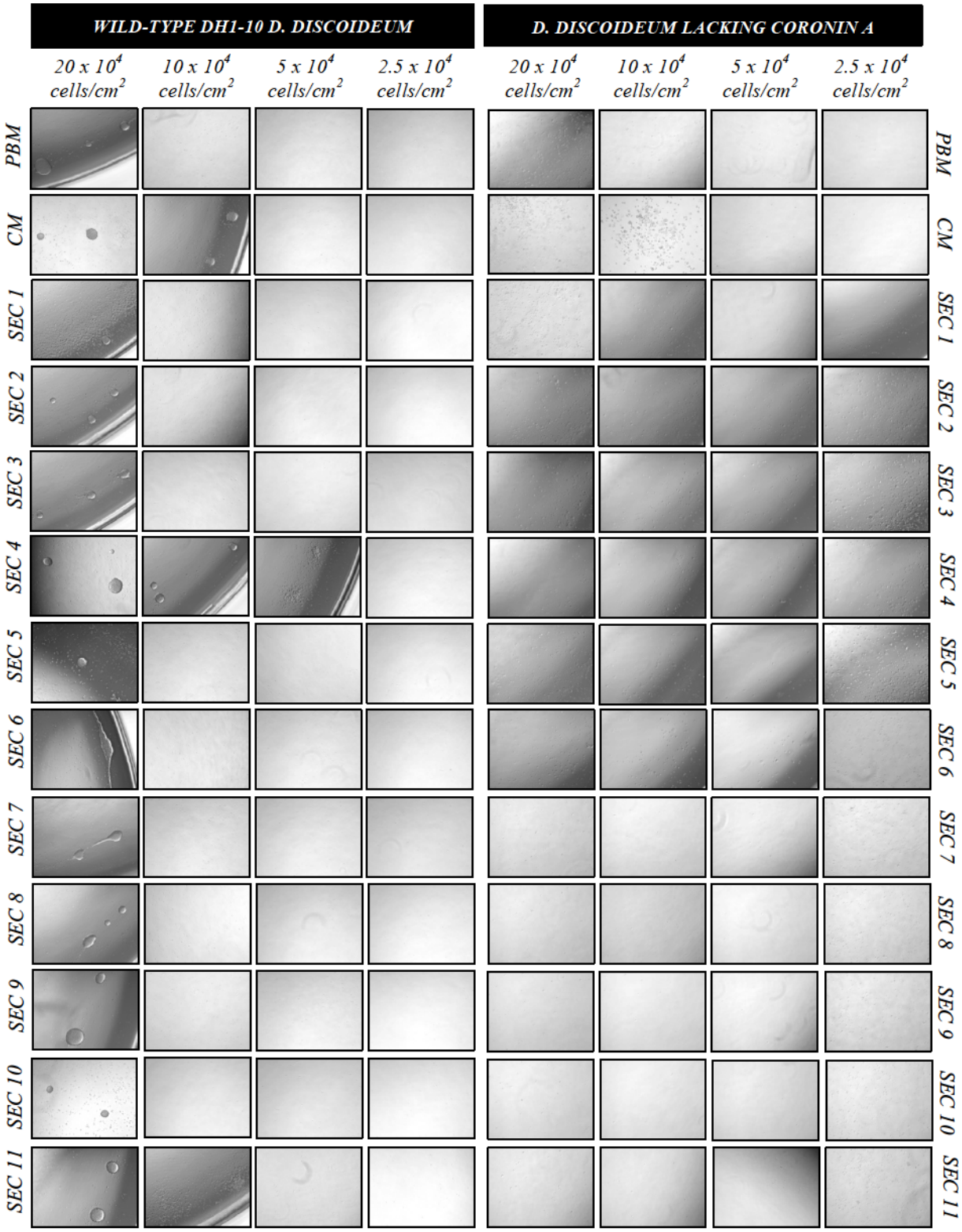


Fig. 18. Cell aggregation in the wild-type DH1-10 strains and coronin A-deficient strains of *D. discoideum*. Both the wild-type and coronin A-deficient cells at the densities of 20, 10, 5, and 2.5 x 10^4 cells/cm^2 were starved in PBM, and then incubated with PBM (negative control), conditioned medium (CM; positive control), flow-through (FT), and elutes from size exclusion chromatography (SEC 1 to 23). Cell aggregation assays were done overnight (16h). *This figure continues on the next page.*

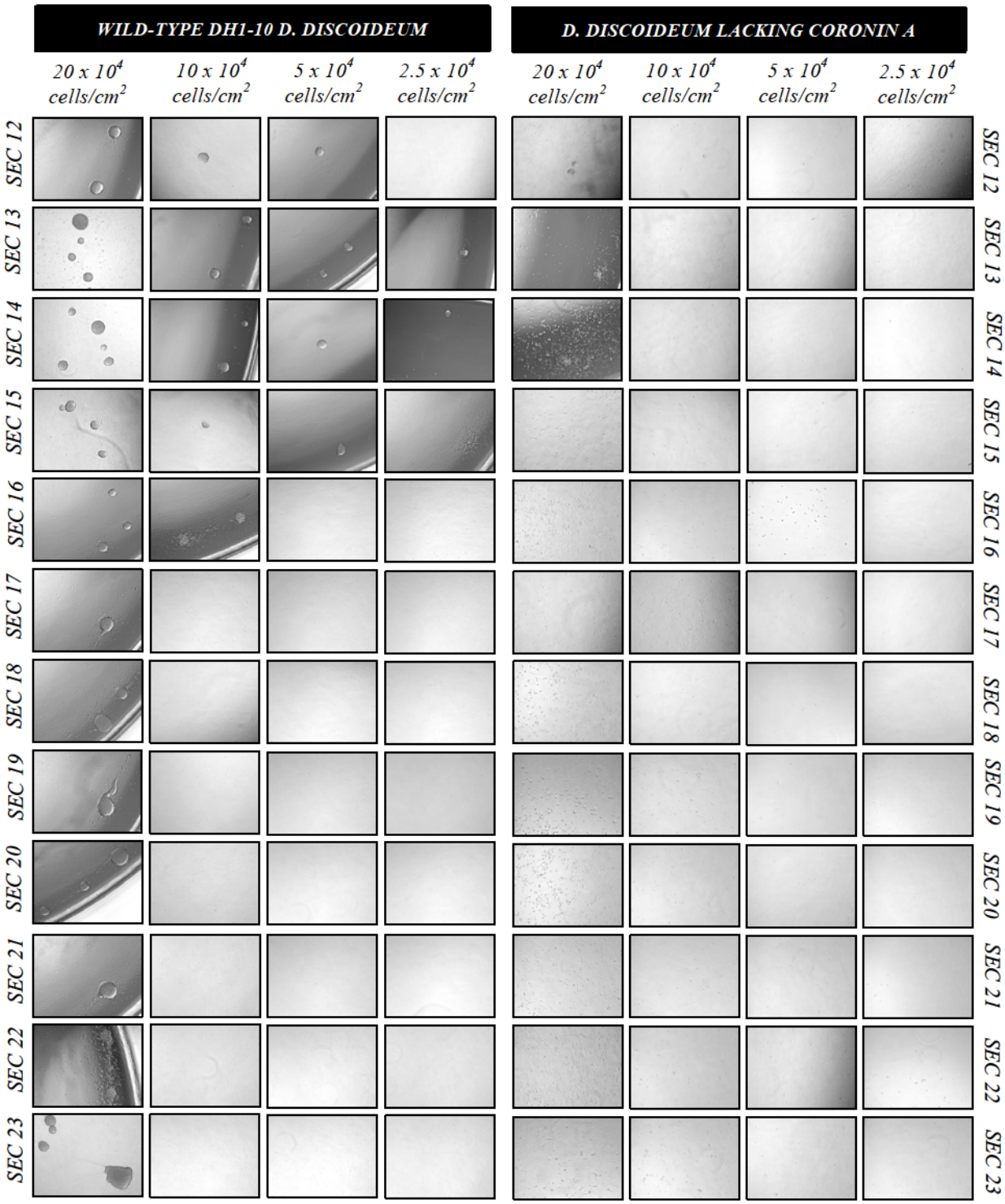


Fig. 18. (*continued*) Cell aggregation in the wild-type DH1-10 strains and coronin A-deficient strains of *D. discoideum*. Both the wild-type and coronin A-deficient cells at the densities of 20, 10, 5, and 2.5 x 10^4 cells/cm^2 were starved in PBM, and then incubated with PBM (negative control), conditioned medium (CM; positive control), flow-through (FT), and elutes from size exclusion chromatography (SEC 1 to 23). Cell aggregation assays were done overnight (16h).

Because effects of the chromatographed factors on the development of cells were reproducible, the results support the functional stability of secreted proteins in cell aggregation experiments. Determining the molecular weight of proteins in SDS-PAGE with silver straining was performed next. Gel electrophoresis of the secreted factor-containing sample did not yield visible bands. To address this issue, we precipitated factors with 10% TCA, then followed by exposure to silver staining. Again, no noticeable bands were observed, and even a long exposure of silver staining did not reveal any bands, suggesting that the protein content in the active fractions was not precipitated during TCA. These results indicate that detection of proteins by silver staining may not be possible, however, mass spectrometry was used on untreated fractions for protein identification and characterization. Due to our repeated attempts to precipitate active fractions using 10% TCA, and during silver staining, we lost most of the active fractions, so conditioned medium sample preparation was carried out again. After conditioned medium was size-fractionated by Superdex 200 following ion exchange chromatography, as described before, the peak fractions (Fig. 19) were examined for the activities. In conditioned medium assays, we found negative cell aggregation responses from the fractions of 6 to 11, and positive responses from those of 12 to 18 (data not shown). To analyze the identity of the different proteins in response to starvation using a Venn diagram, the fractions were subjected to mass spectrometry. As indicated in Fig. 20, conditioned medium from starving *D. discoideum* cells possessed a large number of secreted proteins. Secreted factors in the 6 and 8 negative groups did not induce early development. By comparison, proteins in the 12 and 15 positive groups induced morphogenesis, thus suggesting that factors present in the 6 and 8 negative groups were

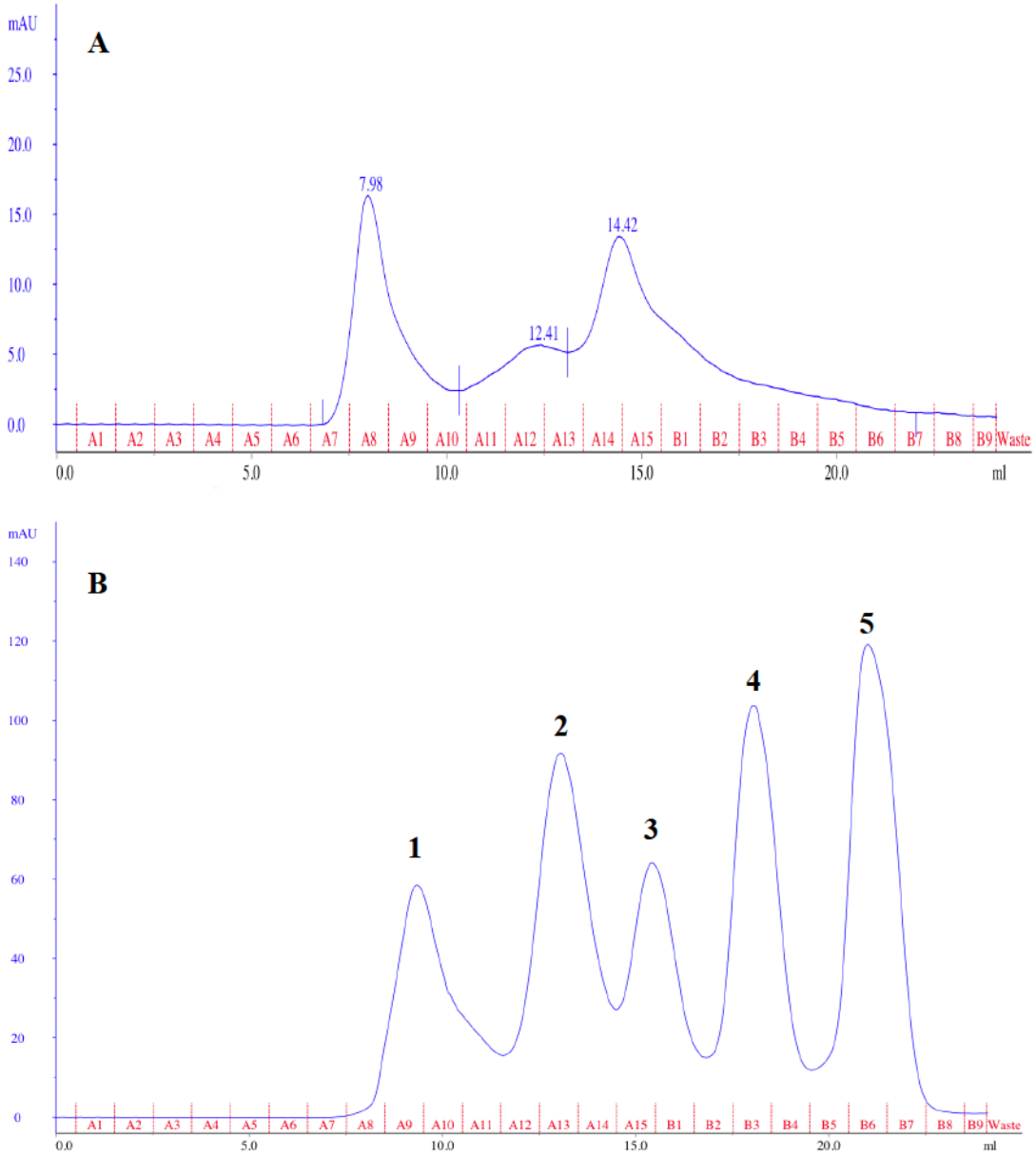


Fig. 19. Further fractionation of active fractions by size exclusion chromatography. **A.** Fractions with cell aggregation-inducing properties eluted after seven ml. Blue line is the milliabsorbance units at A280 nM (mAU); fractions designated as A1 to A15, and B1 to B9, in red, correspond to SEC fraction number of each sample (SEC 1 to SEC 23). **B.** Gel filtration marker standard. Peak 1, 2, 3, 4, and 5 correspond to thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1,350 Da)

excluded as candidate inducers for cell aggregation in a coronin A manner (Fig. 20). As represented in the Venn diagram, 62 proteins, which were overlapped out of 117 and 119 proteins in the 12 and 15 positive groups, were found to be active in response to starvation. Although this number of overlapped proteins is significantly large, we are able to demonstrate that the overlap of 117 proteins with 119 factors result in only eleven interesting hit proteins (dark green) listed in Table 2, indicating a crucial number of secreted factors involved in cell aggregation.

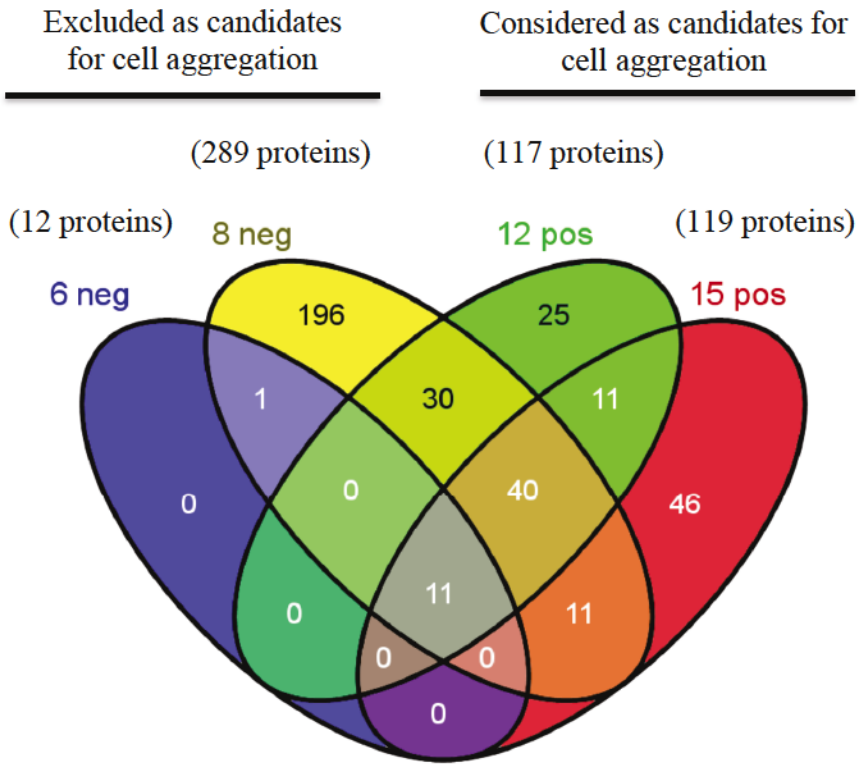


Fig. 20. After mass spectrometry, Venn diagram is used to indicate the overlap between secreted factors analyzed by 6 neg (negative groups; blue), 8 neg (yellow), 12 pos (positive groups; green), and 15 pos (red). Factors with no conditioned medium (CM) activities are assigned in blue (6 neg) and yellow (8 neg) and those with CM activities in green (12 pos) and red (15 pos).

Some of these secreted proteins were examined in further detail since, according to their biological functions, they may be implicated in early development of *D. discoideum*. Thus, to classify accurately the secreted proteins into different functional groups, we inferred their biological roles by using information from different databases available at www.dictybase.org and www.uniprot.org. Information about the secreted factors was furthermore complemented with other information from the literature when it was not available. Table 2 shows a brief description of different functions of the proteins. Several types of proteins have been investigated in respect to their molecular functions: (1) protein(s) required for catalytic/hydrolase activity, and RNA binding; (2) and protein(s) involved in signal transduction. Most molecular and biological functions of the other interesting hit proteins have not been characterized as development-related, such as countin-factor associated protein C, countin-like protein, phosphate starvation-inducible protein, protein psiL, and beta-lactamase-like protein 2. This indicates that the available information about their potential roles in the starvation-induced development is limited. As a result, during starvation the mechanisms of these proteins in the development are not known.

Table 2. List of proteins identified in early *D. discoideum* development

Interesting Hit Proteins	Molecular Functions	Biological Functions
Phospholipase D (PldB)	Catalytic activity	Quorum sensing for cell aggregation
Counting factor-associated protein C (cfaC)	Unknown function	Unknown function
Countin-like protein (ORF Name: DDB_G0274609)	Unknown function	Unknown function
Conditioned medium factor (CMF)	Signal transduction	Cell density sensing and cell aggregation
Phosphate starvation-inducible protein (PsiH)	Unknown function	Unknown function
Sphingomyelinase phosphodiesterase D (sgmD)	Hydrolase activity	Formation of ceramides and macropinocytosis
Protein psiL (psiL)	Unknown function	Macropinocytosis
Communication mutant protein F (comF)	Signal transduction	Cell aggregation during sorocarp development
Cytosol aminopeptidase (lap)	Catalytic activity	Processing intracellular proteins
Probable 39S ribosomal protein L21 (mrpl21)	RNA binding	Protein translation
Beta-lactamase-like protein 2 (ORF Names: DDB_G0280307)	Unknown function	Unknown function

5. DISCUSSION

As described in chapter 4, the conditioned medium (CM) assay suggests that following treatment with starvation medium, coronin A in starving *D. discoideum* cells acts as an essential component in the cell aggregation process that stimulate cells to develop [Vinet et al., 2014]. Since *D. discoideum* cells secrete specific factors into the medium during starvation, this conditioned medium provides a resource for the initiation of an early development [Maeda & Kuwayana, 2000; Mahadeo, & Parent, 2006; Vinet et al., 2014; Loomis, 2014]. As described above, *D.* discoideum cells use these so-called "starvation factors" from the medium to initiate the cAMP signaling pathway, where an intracellular cAMP messenger activates its main target, cAMP-dependent protein kinase A (PKA) [Simon et al., 1989; Insall et al., 1994; Schulkes and Schaap, 1995; Loomis, 2014]. PKA then facilitates the transition from nutrient deprivation-induced starvation to cell aggregation by activating gene expression of developmental genes such as *carA* (cAMP receptor 1) and *aca* (adenylyl cyclase A) [Simon et al., 1989; Insall et al., 1994; Schulkes and Schaap, 1995; Mahadeo & Parent, 2006; Weijer, 2009; Loomis, 2014].

It has been suggested that in *D. discoideum*, cell aggregation strictly depends on the cell density [Hashimoto et al., 1975; Gomer et al., 2011; Ray et al., 2011]. For instance, it was our experience that the cells at a high density were able to aggregate independently of the presence of conditioned medium (PBM buffer used as a negative control) after eight hours of starvation in well plates, whereas the cells at a low density were not able to initiate spontaneous cell aggregation in the absence of conditioned medium. A most likely explanation that accounts for this difference is that during starvation cells at a high density are able to secrete developmental factors, such as

conditioned medium factors (CMFs), that induce these cells to express their cAMP receptors (cAR1s) [Mehdy and Firtel, 1985; Iranfar et al., 2003; Gomer et al., 2011; Ray et al., 2011; Loomis, 2014]. Under this starvation condition, the cells with more cAR1 receptors expressed on their cell surfaces would tend to undergo a state of rapid responsiveness to cAR1 signaling that induces activation of adenylyl cyclase, which in turn produces more cAMP molecules [Iranfar, et al. 2003; Loomis et al., 2014]. As a result, some starving cells aggregate by becoming chemotactic to cAMP, and enter into spontaneous development without requiring addition of conditioned medium [Mehdy and Firtel, 1985; Oohata, 2009; Gomer et al., 2011; Loomis, 2014]. In addition to adenylyl cyclase activation, other critical factors that contribute to cell aggregation are clusters of starving cells, and cell-cell dependent distance [Hashimoto et al., 1975; McCann et al., 2010; Gole et al., 2011; Nagano & Sakurai, 2013]. For example, a cell-cell distance that is less than 100 μm is sufficient for activation of the cAMP relay, but increasing this distance prevents cells from relaying cAMP signals [Gole et al., 2011]. Therefore, for biochemical characterization of our active components in conditioned medium, it was advantageous to subject our laboratory DH1-10 *D. discoideum* and coronin A-deficient strains to deprivation of nutrients using the PBM buffer, and perform a serial dilution of cell densities in order to monitor a certain cell density at which cell aggregation occurs dependently on secreted factors.

In this study, we hypothesized that coronin A initiates cell aggregation in the presence of a specific upstream factor(s) during the starvation response of *D. discoideum*. This discussion focuses on the isolation, purification, and biochemical characterization of an aggregation-inducing factor(s) in conditioned medium (CM) obtained from eight hour-

starved cells. Several biochemical studies have characterized secreted factors in developing *D. discoideum*. Of those, the known factors involved in development are conditioned medium factor (CMF), conditioned medium factor 450 (CMF^{450}), prespore-inducing factor (psi-factor), differentiation inducing factor 1 (DIF-1), cell-counting factor (CF) [Gomer et al., 1991; Gomer et al. 1992; Kay, 1992; Iijima et al, 1995; Oohata et al., 1997; Brock & Gomer, 1999; Roisin-Bouffay, 2000; Brock et al., 2002; Loomis, 2014]. As mentioned in chapter 1, an earlier report indicated that CMF, which is a heat-stable 80-kDa protein secreted in the medium after a few hours of nutrient deprivation, has a crucial role in the early development [Gomer et al., 1991; Gomer et al. 1992; Iijima et al, 1995]. To confirm whether coronin A-dependent cell aggregation regulated by CMF as an upstream factor might fit our hypothetical model (Fig. 21), we heated the whole conditioned medium sample. In this DH1-10 *D. discoideum* strain exposed to heated-medium for 16 hours, it appeared that the activity of conditioned starvation medium was reduced. Whether or not this suggests that the cell aggregation-inducing factor in the medium characterized here is distinct from conditioned medium factor (CMF) remains to be analyzed.

Interestingly, Iijima et al. (1995) reported on another secreted factor, termed CMF^{450}, which differs from CMF in that it is heat-sensitive and a 450-kDa protein. CMF is required for the synthesis of discoidin-I that participates in activation of the cAMP signaling pathway [Iijima et al.,1995; Burdine & Clarke, 1995; Mahadeo & Parent, 2006; Loomis, 2014]. By contrast, CMF^{450} is described to contain a mechanism different from that of CMF, and does not induce the synthesis of discoidin-I, but both CMF and CMF^{450} are essential for the early development [Iijima et al.,1995]. For example, both CMF and

CMF^{450} enhance the ability of developing cells to be responsive to cAMP [Iijima et al., 1995; Ray et al., 2011]. Although CMF^{450} is required for early development, it is possible that it demonstrates the capability to enable developing *D. discoideum* cells to differentiate during late development, whereas CMF is confined to the initiation of development [Iijima et al, 1995; Gomer et al., 1991; Loomis, 2014]. It is important to note that CMF^{450} is secreted after the onset of starvation, however it can be secreted at a high amount after cells starved for 16 hours [Iijima et al, 1995]. Therefore, it is likely that CMF^{450} is secreted in our conditioned medium sample, as it would be conditioned into the medium by *D. discoideum* DH1-10 strains subjected to eight hours of starvation. According to Vinet et al. (2014), conditioned medium factor receptors 1 (CMFR1) specific for CMF are expressed in the strains *D. discoideum* DH1-10. Our heat inactivation results demonstrate that an active component(s), which promotes cell aggregation is unlikely to be CMF, because this factor or the developmental activity of a substance(s) in the conditioned medium was found to be labile to heat treatment.

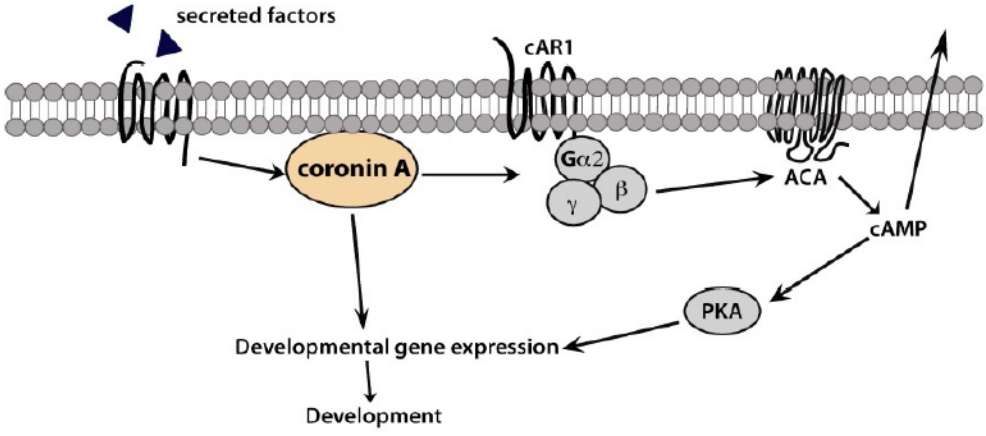


Fig. 21. illustrates a proposed hypothesis for cell aggregation by coronin A via the cAMP signaling transduction. Figure retrieved from Vinet et al., 2014.

If we conclude that wild-type cells do not respond to heated-conditioned medium, our observation is that CMF, which can be detectable in our mass spectrometry analysis, may be destroyed by heat treatment. Our earlier work indicated that a recombinant CMF peptide did not have any developmental effect on starving cells (data not shown and unpublished). Thus, this suggests that our active component examined here seems to be a different-secreted factor(s). Alternatively, our results can be explained by differences in the heat treatment procedure, or breaking down the CMF protein complex into an inactive component.

For other different types of secreted factors(s) in the same conditioned medium (CM) from the eight-hour starved cells, we observed that the heat treatment had a profound effect on the aggregation activity, as observed under the microscope. Overall, we found a significant loss of the conditioned medium activity after heat treatment, suggesting that most secreted factors may be heat-sensitive proteins. However, taking these data based on heat analyses into consideration, we speculate that the heat treatment is probably not based on targeting substances made mainly of only proteins, but also non-proteins such as lipids, amino acids, peptides, or nucleic acids, which all of these diffusible molecules can be conditioned into the medium during starvation [Loomis, et al. 2014]. Therefore, the usefulness of the heat inactivation method characterizing only secreted proteins may be limited since there are other secreted factors that may induce cell aggregation of cells aside from these proteins. Alternatively, to understand more about the molecular properties of active components in the conditioned medium (CM), we tested the effect of the proteinase K treatment on conditioned medium. Many secreted proteins, including CsaA, ctnA, CF, and CMF [www.uniprot.org; www.dictybase.org],

from *D. discoideum* are posttranslationally glycosylated, which is thought to protect them from degradation by either proteases within cell surfaces or extracellular proteases [Hohmann et al., 1987; Brock et al., 2002]. However, in our hands, the proteinase K treatment abolished the conditioned medium activity. The fact that morphogenesis is not induced in wild-type cells by factors after heat treatment and proteolysis, it would appear that they may be considered as proteins, and we show evidence that an active substance(s) involved in regulating *D. discoideum* development is vulnerable to heat and proteolysis.

Interestingly, we observed that treatment of conditioned medium (CM) with a protease inhibitor cocktail significantly inhibited its activity. A proteolytic cleavage of developmental factors appears to be required for migration, and the formation of a cAMP signaling center essential for cell aggregation. Our observation suggests that proteolytic activity might be involved in regulating the development, as suggested by Rossomando et al. (1978), who reported the secretion of extracellular proteases into the conditioned medium at different stages of development. In addition, in agreement with this report, Bakthavatsalam and Gomer (2010) indicated from secreted proteome profiles that developing *D. discoideum* cells released a considerable number of secreted proteinases in conditioned medium, notably after 4 hours of starvation. This reports suggests that in processing of the transition from starvation to cell aggregation, which leads to multicellularity, proteases might induce changes in cell membrane properties in *D. discoideum* cells [Rossomando et al., 1978; Fong & Bonner, 1979]. For example, a protease could release active components from cell membranes during proteolysis, thereby allowing cells to use them to develop into a multicellular structure [Rossomando

et al., 1978; Fong & Bonner, 1979]. In the case of our study, our results suggest that proteases in our eight hour-prepared conditioned medium sample may have more than one significant role in the control of development – for instance, acting on cell membranes and diffusible-secreted substances. Thus, each of them individually can be both secreted factor-target specific, and stage-specific [Rossomando et al., 1978; Bakthavatsalam & Gomer, 2010].

In addition to the role of our studied factor in cell aggregation, a recent study by Fernandez-Lima et al. (2014) has suggested that phosphatidylcholine lipids are present at a high level in the aggregating cells of *D. discoideum*. Furthermore, phospholipase D acts as an enzyme that hydrolyzes phosphatidylcholine to provide the product, phosphatidic acid (PA), which is needed to regulate the CMF signaling pathway [Ray et al., 2011]. Although phosphatidylcholines were not originally found in our starvation medium (CM), it was useful to perform the phospholipase D treatment in order to gain an idea of whether the activity of these lipids have a role in contributing to the aggregation process in the response to the exhaustion of nutrients. To test this, we examined the effect of phospholipase D on the CM sample. It was shown that the cell aggregation activity was variable depending on the cell density in the DH1-10 *D. discoideum* strains. This observation was postulated to be due to the dependence of cell aggregation on both the *D. discoideum* cell density and concentration of phosphatidic acid (PA), suggesting that PA is a source for induction of cell aggregation during starvation [Ray et al., 2011]. An alternative option that we had considered to investigate was that incubating conditioned medium with the other two phospholipases A and C might give different observations on the development [Ray et al., 2011], which was not yet attempted due to the fact that

phosphatidylcholines previously described by Fernandez-Lima et al. (2014) were restricted to the cell membranes in aggregating *D. discoideum* cells rather than to the starvation conditioned medium. Therefore, our phospholipase D analysis did not focus specifically on lipid composition in the plasma membrane, but, rather, was done in order to characterize active molecules in the conditioned medium. Moreover, while it was observed that the conditioned medium activity was variable in wild-type cells after phospholipase D treatment, signal induction for cell aggregation appeared to be most likely due to an artifact in our conditioned medium assays.

The results from the above studies have demonstrated that developmental active factors examined are comprised of proteins, based on their sensitivity to heat treatment and proteolysis. As described in chapter 2, *corA*$^-$ mutants failed to undergo cell aggregation in the presence of conditioned medium [Vinet et al., 2014]. On the basis of this report, we use our current hypothesis model to question whether in response to the starvation coronin A at the plasma membrane senses a certain upstream molecule that acts as a cell-aggregation factor in order to initiate the cAMP signaling pathway essential for the early development in *D. discoideum*. To test this hypothesis, we fractionated the conditioned medium to identify the active factors that induce cell aggregation in a coronin A-dependent manner, and cell aggregation assays of anionic Q fractions given to the wild-type DH1-10 *D. discoideum* strains and mutants lacking coronin A were performed. We observed that several fractions that were active in inducing cell aggregation in the wild-type cells failed to do so in the *corA*$^-$ mutants. Indeed, this confirms our hypothesis that the activity of coronin A is dependent on a cell-aggregation factor(s) for the initiation of morphogenesis. In addition, these results could argue that

some partial cell aggregation activity seen in the *corA*$^-$ cells was due to another development-inducing substance that might induce cell aggregation independently of coronin A. Furthermore, an alternative suggestion is that in this phenotype of *corA*$^-$ mutants there might be some redundancy of chemotactic signaling pathways stimulating these cells to migrate towards a chemoattractant present in the medium. For example, lysophosphatidic acid (LPA) acts as a chemoattractant for starving *D. discoideum* [Jalink et al., 1993; Maciver & Hussey, 2002]. It activates an actin-binding protein, cofilin, indirectly via phosphorylation by LIM kinase [Yang et al., 1998; Noegel & Schleicher, 2000]. Cofilin then accumulates at the leading edge, where it plays a role in allowing cells to initiate chemotaxis, and cell aggregation [Noegel & Schleicher, 2000; Aizawa et al., 2001]. The similar phenotype in *corA*$^-$ mutants can be explained by the fact that cells at a high density (20×10^6 cells/cm^2) can create a short cell-cell distance between them, which is necessary for cell aggregation if cells are close enough to each other in response to cAMP signals [Gole et al., 2011; Nagano & Sakurai, 2013]. In conditioned medium experiments, we observed that *corA*$^-$ mutants delayed their development (cell aggregation) at a high cell density and failed to aggregate at a low density, whereas wild-type cells were able to aggregate at a density of 20×10^6 to 2.5×10^6 cells/cm^2.

Having confirmed our hypothesis that coronin A requires activation by a specific-secreted factor(s) during the early development, we wanted to further identify substances in the conditioned medium sample in order to isolate a coronin A-dependent cell aggregation factor. Eluted fractions in SDS-PAGE gels stained with silver staining resulted in the visualization of many protein bands, having have molecular weights in the range from 30 to 130 kDa. The difference in the molecular weights of secreted factors is

likely due to posttranslational glycosylation, as well as to distinct protein sizes (high molecular weight bands) [Brock et al., 2002; www.dictybase.org; www.uniport.org]. It is noteworthy to point out that the bands of secreted factors at the early development stage are much similar in size. This suggests that the secretion of such similar proteins is essential to sustain cell aggregation, and fractionation of conditioned medium by ion exchange chromatography could lead to purification of cell aggregation factors, where the cell aggregation activities of fractions were increased as compared to untreated conditioned medium (positive control). This suggests that the impurities were removed by the anion exchange column. We demonstrate that combination of purification approaches with ion exchange chromatography, gel filtration chromatography, and mass spectrometry has a potential to enhance purification of whole conditioned medium.

Our work demonstrate that we are able to isolate a number of secreted proteins in conditioned medium after ultrafiltration, ion exchange and size exclusion columns, and mass spectrometry. By using these approaches, we identified eleven secreted proteins related to development, such as: phospholipase D (PldB), counting factor-associated protein C (cfaC), countin-like protein, conditioned medium factor (CMF), phosphate starvation-inducible protein (PsiH), sphingoomyelinase phosphodiesterase D (sgmD), protein psiL (psiL), communication mutant protein F (comF), cytosol aminopeptidase (lap), probable 39S ribosomal protein L21 (mrp121), and beta-lactamase-like protein 2. So far, only five proteins involved in different stages of development have been described in the literature. Among them, conditioned medium factor (CMF), phospholipase D (PldB), and sphingomyelinase phosphodiesterase D (sgmD) are required for early development, while communication mutant protein F (comF), and cytosol

aminopeptidase (lap) participate in late development, particularly spore formation and differentiation [Gomer et al., 1991; Gomer et al., 1992; Li et al., 2001; Kibler et al., 2003; Ray et al., 2011; Poloz et al., 2012]. Although mass spectrometry identified communication mutant protein F and cytosol aminopeptidase as later development-related factors, our description was mainly focused on conditioned medium factor, phospholipase D, and sphingoomyelinase phosphodiesterase D, because they have been involved in regulating cell aggregation through the cAMP signaling pathway [Gomer et al., 1991; Gomer et al., 1992; Machwate, et al., 1998; Li et al., 2001; Ray et al., 2011]. Ray et al. (2011) demonstrates that the binding of CMF to its receptor inhibits phosphatidic acid production by inactivating phospholipase D, which results in the dissociation of the Gα2 from βγ subunits. This leads to an increase in cAMP levels that allow for chemotaxis and cell aggregation [Ray et al., 2011]. Some work from the literature provides evidence that in *D. discoideum* sphingomyelinase phosphodiesterase D is involved in cell aggregation, through which it produces ceramide molecules required by ceramidase for formation of sphingosine lipids [Li et al., 2001; Hannun & Obeid, 2008].

As observed before, as the activity of conditioned medium (CM) became unstable after heat and protease treatment, and because of absence of cell aggregation in wild-type cells, it would be unlikely that CMF is a candidate for the cell aggregation-inducible factor in our cell system. We speculate that CMF, at least on its own, might not be the only component that activates the cAMP signaling pathway, and that other proteins are required in concert with CMF for activation of the cAMP relay. The best candidate for a secreted factor to mediate the induction of cell aggregation in a coronin A-manner would

be sphingomyelinase phosphodiesterase D, as the properties of this protein have been described in the literature [Li et al., 2001; Hannun & Obeid, 2008; Usatyuk et al., 2013]. For example, sphingosine, the cleaved metabolite of ceramide, is phosphorylated by a sphingosine kinase to bind its specific G-protein coupled S1P1-5 receptor as sphingosine-1-phosphate (S1P). This activated receptor activates phospholipase D2, which produces phosphatidic acid (PA) by hydrolyzing cytosolic membrane phosphatidylcholine. PA then activates proteinase kinase C zeta (PKC ζ) that allows coronin 1B protein to initiate chemotaxis and cell migration. However, this observation was reported in human pulmonary artery endothelial cells (HPAECs), thus the involvement of sphingoomyelinase phosphodiesterase D has not been studied in *D. discoideum* [Usatyuk et al., 2013]. Interestingly, other work demonstrated that sphingosine-1-phosphate (S1P) regulates cAMP levels in *D. discoideum* via inactivation of adenylate cyclase, and this can be explained if levels of S1P increase [Machwate, et al., 1998; Brocklyn et al., 1998]. However, when conditions are favorable for *D. discoideum* development, levels of cAMP increase, which can in turn inactivate sphingosine kinase in order to regulate the S1P levels [Machwate, et al., 1998; Brocklyn et al., 1998]. Therefore, it would be of interest to characterize *D. discoideum* sphingoomyelinase phosphodiesterase D, and determine the relationship between this secreted factor and cAMP levels in starving cells, where regulation of protein kinase A (PKA) activity by cAMP would be expected to affect morphogenesis.

In this study, the other interesting proteins identified by mass spectrometry that might be involved in cell aggregation during early development are countin factor-associated protein C (cfaC), countin-like protein, phosphate starvation-inducible protein

(PsiH), protein psiL (psiL), and beta-lactamase-like protein 2. Nevertheless, to the best of our knowledge, functions of these proteins have not been well defined, and their protein identification in Table 2 was based on peptide sequence similarity detected by mass spectrometry. In the gel filtration column, the order of elution of fractions is based on size, and we observed an earlier elution of a fraction with a high molecular weight (see gel filtration marker standard), which would correspond to a multisubunit factor complex named by Brock and Gomer (1999) as a counting factor with a molecular weight of about 450 kDa. When this 450 kDa-counting factor is purified, it reveals eight different proteins [Brock et al., 2002; Choe et al., 2009]. Although not evident, it is possible that this counting factor is present in the conditioned medium sample from cells that remained starved for eight hours, as confirmed by a secretome analysis [Bakthavatsalam & Gomer, 2010]. Brock and Gomer (1999) suggests that in order for the factor to bind to its receptor to regulate aggregate sizes, it requires the presence of other polypeptides for receptor binding. Thus, this raises the possibility that countin factor-associated protein C (cfaC) and countin-like protein could form a part of the multisubunit counting factor.

In summary, our findings described above suggest that secreted factors required for coronin A-dependent cell aggregation are sensitive to heat and protease treatment, and can be of distinct molecular weight. It is apparent from the results of our present study that the active factors from conditioned medium (CM) are important for activation of coronin A in the *D. discoideum* cells during the transition from starvation to cell aggregation. *corA*$^{-}$ mutants incubated with active fractions demonstrated that they failed to aggregate or delayed their development. We showed that our purification approaches enabled us to narrow down a large number of secreted factors to few proteins, which can

be studied as candidates for the cell aggregation inducible factors dependent on coronin A. Although thus far several secreted factors proposed in Table 2 to be responsible for activating coronin A in response to the starvation are coronin A-dependent proteins, it remains to be determined which specific protein is involved in mediating the cell aggregation pathway in a coronin A-dependent manner. Presently, we are preparing more conditioned medium for further candidate identification and characterization.

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Curriculum Vitae

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EDUCATIONAL BACKGROUND

Master of Science, **Biotechnology**, Johns Hopkins University, Zanvyl Krieger School of Arts and Sciences, Baltimore, Maryland, USA. 2011 – May 2015

Master Thesis title: "The Activity of Coronin A is Dependent on a Protein(s) Secreted by *Dictyostelium Discoideum* in the Early Starvation Response"

Bachelor of Science, **Chemistry**, Gallaudet University, Washington, DC, USA. 2002 – 2007

UNDERGRADUATE COURSEWORK

Biochemistry, Biology I and II (+lab), General Chemistry I and II (+lab), Research Methods in Biology, Organic Chemistry I and II (+lab), Advanced Inorganic Chemistry, Analytical Chemistry I and II (+lab), Physical Chemistry I and II (+lab), Pharmacology, Physics I and II (+lab), Pre-calculus and Calculus I.

GRADUATE COURSEWORK

Johns Hopkins University, Zanvyl Krieger School of Arts and Sciences, Baltimore, Maryland, USA

Biochemistry, Molecular Biology, Advanced Cell Biology I and II, Epigenetics: Gene Organization and Expression, Principles of Immunology, Pathogenic Bacteriology, Molecular Basis of Pharmacology, Stem Cell Biology, Independent Research in Biotechnology, and Biotechnology Thesis

OTHER GRADUATE COURSEWORK

Georgetown University Medical Center, Department of Pharmacology and Physiology, Washington, DC, USA

Principles of Pharmacology, Fundamentals of Human Physiology, and Biostatistics

RELEVANT WORK EXPERIENCE

Independent Research in Biotechnology, and Biotechnology Thesis
Biozentrum, University of Basel, Basel, Switzerland
Coronin A signaling in the development of Dictyostelium discoideum during the early starvation response. Jan 2014 – Dec 2014

Cancer Research Training Award (CRTA)
Laboratory of Cancer Biology and Genetics, Center for Cancer Research National Cancer Institute (NCI), Bethesda, Maryland, USA.
Characterization of phorbol ester-protein kinase C (PKC) interactions in different human cancer cell lines. Aug 2009 – June 2011

Pulmonary Hypertension Research Assistant
Department of Pharmacology and Physiology, Georgetown University Medical Center, Washington, DC, USA
Conducted testing for confirmation of binding CBF/NFY to CCAAT box inside the cloned GATA-4 promoter in the HL-1 cardiac muscle cells.
Fall 2008 (five months)

Research Experiences for Undergraduates (REU)
Department of Chemistry and Biochemistry, James Madison University, Harrisonburg, Virginia, USA
Research focused on how RecA protein structure and function in E. coli are regulated by nucleotide binding (ATP and ADP) and salts. Summer 2007 (three months)

FELLOWSHIPS, HONORS AND AWARDS

Cancer Research Training Award (CRTA) fellow, funded by National Institutes of Health (NIH) in the US. 2009 – June 2011

Animal and Human Cell Culture: Method and Applications Certificate, by Foundation for Advanced Education in the Sciences graduate (FAES) school at National Institutes of Health (NIH). Summer 2010 (one month)

Research Experiences for Undergraduates (REU), funded by US National Science Foundation, $ 3,000. Summer 2007 (three months)

Horse Care and Training Certificate, by Spain Centro Ecuestre Jerezano. Summer 2003 (three months)

Fantastic Student of Year. Fall 2003

Dean's List. 2003 and 2007

Swindells Endowment, $ 2,000. 2003 – 2004

Grant-in-Aid, $ 2,000. 2002 – 2003

Zoo Animal Care Certificate, by Spain Centros de Recuperación de Animales Silvestres (Center for Wild Animals Rehabilitation). 1998 – 2001 (only summer).

EQUIPMENT SKILLS AND LABORATORY EXPERIENCE

Trained in the use of pH meter, electron and confocal microscopy, centrifuge, autoclave, and spectrophotometer. Experienced in techniques of High-Performance Liquid Chromatography (HPLC), Infrared Spectroscopy, Chromatin Immunoprecipitation (ChiP), Circular Dichrosim (CD), Polymerase Chain Reaction (PCR), cell culture, UV Spectroscopy, immunoblotting (Western Blotting), SDS-PAGE, electrophoresis, chromatographic techniques (ion exchange and size exclusion chromatography), isolation and purification of protein and DNA

COMPUTER SKILLS

Microsoft Word, Excel, PowerPoint, Adobe Photoshop and Aperture (Mac)

PRESENTATION

Research Experiences for Undergraduates (REU) presentation titled as "Circular Dichroism Studies of Salt Effects on the Unfolding of the RecA Protein"

POSTERS

Kedei, N., Telek, A., **Lubart, E.S.**, Lewin, N.E., Yang, D., Chen, J., Herrmann, M., Goldsmith, P.K., Lim, L., Mannam, P., Garfield, S.H., Blumberg, P.M. Diverse Patterns of Biological Response to Phorbol Esters and Related Protein Kinase C Activators in LNCaP Human Prostate Cancer Cells. 102th Annual meeting of American Association for Cancer Research (AACR), Orlando, Florida, USA, 2011

PUBLICATIONS

Noemi Kedei, Andrea Telek, Alexandra Czap, **Emanuel S. Lubart**, Gabriella Czifra, Dazhi Yang, Jinqiu Chen, Tyler Morrison, Paul K. Goldsmith, Langston Lim, Poonam Mannan, SusanH. Garfield, Matthew B. Kraft, Wei Li, Gary E. Keck, Peter M. Blumberg; the Synthetic Bryostatin Analog Merle 23 Dissects Distinct Mechanisms of Bryostatin Activity in the LNCaP Human Prostate Cancer Cell Line. Biochemical Pharmacology. 10858: 1–13, 2011

Noemi Kedei, **Emanuel Lubart**, Nancy E. Lewin, Andrea Telek, Langston Lim, Poonam Mannan, Susan H. Garfield, Matthew B. Kraft, Gary E. Keck, Sofiya Kolusheva, Raz Jelinek, Peter M. Blumberg; Some Phorbol Esters Might Partially Resemble Bryostatin 1 in their Actions on LNCaP Prostate Cancer Cells and U937 Leukemia Cells. ChemBioChem. 0000, 00, 1 – 11, 2011

AFFILITATIONS

English Language Institute Students Organization (ELISO), 2001 – 2006

LANGUAGES

Fluent in English and Spanish. Conversant in German – A2 level

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References

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