The Dictyostelium discoideum Rap1 signalling cascade and its functions during growth and development

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CHAPTER VII
Summary

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Summary

Over the last three decades the small G-protein Rap has been the focus of numerous studies. Early research on Rap proteins was mostly focused on their ability to revert the oncogenic ras transforming phenotype (Cook et al., 1993). Nowadays they are recognized to be important regulators of cell polarity, substrate and cell-cell adhesion, and other processes that involve regulation of cytoskeletal rearrangements (Zwartkruis and Bos, 1999). However, despite extensive studies in various biological systems, including Dictyostelium, yeast, nematodes, fly, mice and human cells, the Rap1 signalling pathways are still not completely identified and characterized (Frische and Zwartkruis, 2010).

Because of its good genetical tractability and high evolutionary conservation of both Rap protein sequence and the signalling pathways that govern cytoskeletal rearrangements, Dictyostelium serves as an excellent model for studying Rap1 dependent processes (Chubb and Insall, 2001). Dictyostelium Rap1 is crucial for growth, chemotaxis and multicellular development (Rebstein et al., 1993; Kortholt and van Haastert, 2008; Parkinson et al., 2008). In this thesis I have identified several new Rap effectors and regulators. The signalling pathways that govern Rap1 activity and the subsequent downstream molecular cascades have high levels of complexity and together allow for an important function of Rap1 throughout the Dictyostelium life cycle.

So far seven regulatory proteins have been identified that tightly control Rap1 activity. In chapter 1 we showed that GbpD and GefQ are responsible for activation of Rap1 during the vegetative state, while GefL is the major regulator during later stages of Dictyostelium development (Chapter 2). The deactivation of Rap1 is regulated by GAP proteins: RapGAP1 acts at the front of the extending pseudopods during chemotaxis (Jeon et al., 2007). RapGAP2 and RapGAP3 proteins have similar functions in regulating multicellular development (Jeon et al., 2009; Parkinson et al., 2009), while RapGAP9 seems to regulate Rap activation in both vegetative and starved cells (Mun et al., 2014). Together this shows that despite partial overlap of functions most of the GEFs and GAPs appear to regulate separate pathways, kept apart in time and space. The differences in subcellular localization of the GEF and GAP proteins, their gene expression times, and posttranslational modifications are likely contributing to diversity of Rap1 regulators functions.
Rap1 functions mainly in controlling cytoskeleton rearrangements; it stimulates cellular adhesion, actin filament formation, and myosin disassembly (Kortholt and van Haastert, 2008; Mun and Jeon, 2012). Vegetative amoeba cells constantly change their shape and strength of substratum adhesion: they chase the bacteria by folic acid mediated chemotaxis, they form membrane cups needed for the phagocytosis and macropinocytosis, and finally they undergo cell division to multiply (Bozarro and Eichinger, 2011; Bonner, 1999). Disturbing Rap1 signalling, by expressing the constitutive active Rap1G12V variant of the protein, leads to a strongly increased cellular adhesion and phagocytosis, suggesting that Rap1-mediated adhesion plays an important role during vegetative growth. Previously it was shown that Phg2 (Kortholt et al., 2006) and PI3K (Kortholt et al., 2010) are primarily regulating adhesion during the vegetative state of the cells.

Next to its role in vegetative growth, adhesion is also crucial for proper morphogenesis. Starved cells chemotax towards aggregation centres, and move within multicellular aggregates to form patterns of cells with different fates of either becoming dead stalk cells or dormant spores (Bonner, 1999). Less was known about how Rap1 activity affects cellular adhesion and force transmission during that process. We showed that the interaction between activated Rap1 and TalinB is crucial for efficient morphogenesis (Chapter 4). We further showed that although Rap1 does not bind to TalinA, it can stimulate TalinA mediated substratum adhesion (Chapter 4). We speculate that this is due to the potential crosstalk between Rap1 effector Phg2 and TalinA (Gebbie et al., 2004).

Phg2 is also essential for Rap1 mediated myosin disassembly; Phg2 kinase directly (or indirectly) phosphorylates Myosin Heavy Chain Kinase (Jeon et al., 2007). Phosphorylated myosin does not assemble in filaments, therefore active Rap1, through Phg2, negatively regulates myosin assembly (Jeon et al., 2007). Additionally to its function in regulating myosin, Rap1 also stimulates actin polymerization via activation of Rac family proteins. Only two years ago the first report of the direct interaction between activated Rap1 and RacGEF1 protein shed light on this phenomena (Mun and Jeon, 2012). In our work we show that this mechanism may be used by Dictyostelium also in regulating multicellular development. A RacGEF GxcC, which is crucial for proper Dictyostelium development, showed to specifically interact with active Rap1 (Chapter 3).

Cytoskeletal rearrangements play an important role during both chemotaxis and cytokinesis. Consistently, previous results have shown that Rap1 indeed plays a role
during chemotaxis towards cAMP: Rap1 is locally activated at the migrating cells’ leading edge and Rap1 hyperactivation results in severe chemotaxis defects (Kortholt and van Haastert, 2008; Jeon et al., 2007). Our data allows for better understanding of the role that Rap1 plays in regulating chemotaxis: mutants cells that fail to activate Rap1 at the leading edge move with a decreased speed and chemotaxis index towards both folate and cAMP (Chapter 2). However, since these cells are still able to sense and move towards the chemoattractant, although less efficient, Rap1 is not part of the core gradient signalling pathway, but rather acts in amplifying the signal. Its roles may include stimulating actin filament formation at the cell front, regulating cellular adhesion and restricting myosin filament formation to the rear of the cell (for detailed review of Rap1 functions during chemotaxis see Lee and Jeon, 2012 and Chapter 2).

In chapter 6 we show that also in the process of cytokinesis progression Rap1 activity is tightly spatiotemporally controlled. In dividing cells that did not start the cytokinesis process yet, Rap1 is activated uniformly around the cell membrane, and during cytokinesis Rap1 has clear polar activation pattern (Plak et al., 2014; Chapter 6). This tight regulation of spatiotemporal Rap1 activity is essential for the orchestrated regulation of three cytoskeletal components during cell division, actin, myosin and tubulin (Chapter 6). Cell division starts with the rounding up phase, when cells decrease their adhesion strength and rearrange their actin and myosin cortex cytoskeleton (Lancaster and Baum, 2014). The initial decrease in Rap1 activation, and the uniform structure of it, allow for the slight detachment from the substrate, and the subsequent formation of a uniform acto-myosin cortex during the step of the spindle assembly. Once the spindle is assembled, polarised signals from either the mitotic spindle, or dividing nucleus lead to polar activation of Rap1 (Chapter 6). This pattern allows for myosin filament formation only at the sites with lowest Rap1 activity (cell furrow), where at the same time the adhesion strength is lowest. Furthermore Rap can stimulate the active cell crawling of the polar regions by stimulating actin filament formation, therefore further facilitating the scission of daughter cells. Interestingly, Rap1 not only regulates the actin/myosin cytoskeleton but Rap1 hyperactivation also results in severe microtubule defects (Chapter 6).

Although, rap1 is considered to be an essential gene for Dictyostelium, so far no effector protein was discovered that couldn’t be knocked-out. There are two possible explanations to this phenomena: first, there may be a so far undiscovered effector, that acts downstream of Rap1 and is essential for cell viability. It is unlikely, however not
impossible, that despite over 20 years of studies we are still missing this crucial link. The other explanation is based on the results published in this thesis, showing Rap1 to act as a global regulator of a large number of processes crucial for Dictyostelium, including actin, myosin and microtubule filament formation, adhesion and protrusion formation. We suspect that in the absence of Rap1 all these processes are severely affected and that together explains why Rap1 is essential. Therefore not one but a number of effector genes must have been deleted to achieve rap1 knock out-like phenotype.

Altogether our results show that Rap1 acts as a global regulator of cytoskeletal changes, playing a role in almost any cellular or morphogenetic process in Dictyostelium life cycle. The data presented here will further help to resolve many of the existing questions in Dictyostelium cell biology. It also opens a way to forming new hypotheses and designing experiments. One of the interesting possibilities will be the use of Rap1 activation-deficient mutants in studying core signalling pathways in chemotaxis. Taking Rap1 activity out of the picture may help to better understand the crosstalk between actin filament formation and Ras activity, a problem that is still extensively debated in Dictyostelium (Kortholt et al., 2011, Srinivasan et al., 2013). Another interesting possibility is the use of Dictyostelium cells to better understand evolution of cell division and cytokinesis process. The data from SCAR mutants (regulators of branched actin filament formation) (King et al., 2010) and our data about Rap1 activity during cytokinesis (Chapter 6) strongly suggest that active crawling may contribute to efficient division of adherent amoeboid cells. At the other hand most of the higher eukaryotic cells are proposed to divide in process dependent on subsequent equatorial contraction and polar relaxation, thus processes at the cell poles are passive. Therefore it will be interesting to study the difference between the two systems, and see how the two cytokinesis strategies evolved.

We hope that our work opens the door for new experiments and ideas and will promote the use of Dictyostelium as a model organism in studying Rap1 dependent cytoskeleton dynamics.
References


Samenvatting

Elk levend organisme, of het nu een enkele cel is of een verzameling van miljoenen cellen die samen gespecialiseerde weefsels of organen vormen, kan per definitie basale processen uitvoeren. Omdat basale functies, zoals voedselopname en -vertering, celgroei en celdeling alleen plaats mogen vinden onder bepaalde omstandigheden, moeten deze processen onderling exact gecoördineerd worden in tijd en ruimte. Groei en deling vinden bijvoorbeeld alleen plaats in een voedselrijke omgeving. Coördinatie wordt gereguleerd door signaleringsroutes in de cel die snel aan en/of uit geschakeld kunnen worden als reactie op interne of externe stimuli. Op eiwit niveau kan dit aan/uit schakelen bereikt worden door het toevoegen of verwijderen van een functionele groep (zoals (de)fosforylering en ubiquitinering), door conformatieveranderingen in het eiwit, door het vormen van eiwit-eiwit complexen, of door een verandering in lokalisatie van het eiwit.

Ras is een verzamelnaam voor een familie van signaleringseiwitten welke in vrijwel alle eukaryoten voorkomt en evolutionair sterk geconserveerd is. Een Ras eiwit is gebonden aan óf een GTP- óf een GDP-molecuul, wat de actieve, respectievelijk inactieve toestand weerspiegelt. Alleen in de actieve vorm bindt Ras aan zijn effectoren en zet daarmee de onderliggende signaleringscascades aan. Ras activatie (GTP binding) is afhankelijk van de aanwezigheid van andere eiwitten, GEF’s (Guanine Exchange Factors), die het uitwisselen van een GDP molecuul voor een GTP molecuul versnellen. Deze activatie is reversibel omdat naast GEF’s ook GAP eiwitten voorkomen, die de afbraak van GTP (hydrolyse) versnellen, wat weer resulteert in inactief Ras en het uitzetten van de cascade. Ras eiwitten zijn dus zeer efficiënte moleculaire schakelaars, die een belangrijke rol spelen in de regulatie van veel cellulaire processen.

Het doel van dit proefschrift was om de functie van een van deze Ras eiwitten, Rap1, in meer detail te onderzoeken. Als modelsysteem hebben we hiervoor het organisme Dictyostelium discoideum gebruikt. In voedselrijke gunstige omstandigheden leeft Dictyostelium als een eencellige amoebe (vegetatieve groei), maar onder slechte omstandigheden, zoals bij voedselschaarste, gaan de cellen in het meercellig ontwikkelingsstadium. Voedselgebrek induceert een sterke verandering in de gen expressie en in de productie en secretie van cAMP; de naast gelegen cel signaleert het
cAMP en beweegt ernaar toe (chemotaxis) wat resulteert in de vorming van een meercellig organisme (aggregaat). De cellen in het aggregaat differentiëren tot sporen of tot ondersteunende cellen (“stalks”); uiteindelijk is een cluster van sporen gevormd bovenop een cellulose steeltje van 1-2mm hoog.

Wij hebben Dictyostelium gebruikt voor ons onderzoek omdat het Rap1 eiwit en de onderliggende signaleringsroutes in dit organisme grotendeels evolutionair geconserveerde met de menselijke variant, terwijl het technisch veel makkelijker is om experimenten te doen met Dictyostelium dan met humane cellen.

Het eerste deel van dit proefschrift (hoofdstuk 2) behandelt de regulering van Rap1 activiteit. Tot voor kort was het onderzoek toegespitst op GAP eiwitten die de Rap1 signalering afremmen. Hier beschrijven we twee nieuwe GEF eiwitten in Dictyostelium die Rap1 activeren tijdens vegetatieve groei (GefQ) en als meercellig organisme (GefL). We vergelijken de functie van GefQ en GefL met de functie van GbpD-GEF, de reeds bekende activator van Rap1. We tonen aan dat tijdens vegetatieve groei de functies van GbpD en GefQ elkaar gedeeltelijk overlappen en dat beide eiwitten betrokken zijn bij de regulatie van adhesie, groei en beweging. GefL is actief bij de regulatie van processen die door voedselgebrek geïnduceerd worden, zoals chemotaxis en ontwikkeling naar meercellig organisme.

Het tweede deel van dit proefschrift, bestaande uit de hoofdstukken 3, 4 en 5 is toegespitst op het vinden van eiwitten die specifiek binden aan actief Rap1. Dit is op twee manieren gedaan: een proteomics aanpak met opgezuiverd recombinant Rap1 eiwit en Dictyostelium cellen, en daarnaast hebben we in de Dictyostelium database gezocht naar eiwitten met een mogelijk Rap1-bindingsmotief. Op deze manier hebben we een groot aantal mogelijke Rap1-interactie partners gevonden; echter, voor een groot aantal van deze hits moet de Rap1-binding nog experimenteel geverifieerd worden. De interactie van TalinB en GxcC met Rap1 is geverifieerd en gedetailleerde analyse toont aan dat deze Rap1 bindende eiwitten noodzakelijk zijn voor de ontwikkeling van Dictyostelium als meercellig organisme.

In het laatste deel beschrijven we een nieuwe functie van Rap1, namelijk regulering van celdeling (cytokinese). Bij het opsplitsen van de moedercel in twee dochtercellen is Rap1 specifiek geactiveerd op de twee polen van de cel. Wij tonen aan dat dit essentieel is voor de regulatie van het arrangeren van actine en myosine in het
cytoskelet en voor adhesie. Beide processen zijn essentieel voor het ontstaan van de specifieke cel vorm die resulteert in een normale (symmetrische) celdeling.

Samenvattend laten de data in dit proefschrift zien dat Rap1 een belangrijke regulerende functie heeft bij een veelheid van cellulaire processen. Tijdens vegetatieve groei reguleert het cel beweging, voedselopname, substraat adhesie en celdeling, terwijl in gehongerde cellen het Rap1 betrokken is bij het dirigeren van processen als chemotaxis en ontwikkeling naar meercellig organisme. Onze conclusie is dat Rap1 beschouwd kan worden als centrale component in verscheidene essentiële signaleringscascades in Dictyostelium.
This is really the end. And it was a long journey. What is left is to say thank you to all who made it possible for me to go through the time of my PhD studies. Thank you for all the help and support.

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