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Dissertation

Cloning and characterisation of a *Dictyostelium* STE20-like protein kinase DST2

Hyunju Son

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DST2, a *Dictyostelium* STE20-like kinase, is phosphorylated by PKA and phosphorylates the actin binding protein severin. The American Society for Cell Biology (ASCB) 41st Annual meeting abstract, December 2001.

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European Life Scientist Organisation (ELSO) 1st Annual meeting abstract, September 2000.

The experimental parts of the work presented here were carried out in the laboratory of Prof. Dr. M. Schleicher from May 1999 to December 2001 at the Adolf-Butenandt-Institute of cell biology, Ludwig-Maximilians-universitaet Muenchen.

Table of Contents

ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
bp	Base pair(s)
BSA	Bovine serum albumin
C-	Carboxy-
cDNA	Complementary DNA
CIP	Calf intestinal phosphatase
conc	Concentrated
D	Dalton
DNA	Desoxyribonuleic acid
dNTP	Desoxiribonucletide triphosphate
DNase	Deoxyribonuclease
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylene-diamine-tetraacetic acid
EGTA	Ethylene glycol-bis-(2-aminoethylether)-N,N'-tetraacetic acid
et al	And others
Fig.	Figure
F-actin	Filamentous (polymerized) actin
FITC	Fluorescein isothiocyanate
G-actin	Globular actin (monomer)

GFP	Green fluorescent protein
h	Hour(s)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
His-tag	Histidine-tag
Hs	*Homo sapiens*
IgG	Immunoglobulin G
ITPG	Isopropyl-ß-thiogalactopyranoside
kb	kilo base(s)
kDa	Kilodalton
l	Litre(s)
LMW	Low molecular weight
µ	Micro
M	Mol/l
mAb	Monoclonal antibody
min	Minute(s)
ml	Mililitres
µm	Micrometer
mM	Milimolar
MOPS	Morpholinopropanesulfonic acid
nm	Nanometer
N-	Amino-
NADH	Nicotine adenine dinucleotide
OD	Optical density

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIP_2	Phosphatidylinositol-4,5-diphosphate
PMSF	Phenylmethylsulfonyl fluoride
pH	Negative decadic logarithm of proton concentration
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Room temperature
s, sec	Seconds(s)
SDS	Sodium dodecyl sulfate
TBE	Tris/borate/EDTA
TBS	Tris buffered saline
TEMED	N,N,N',N'-tetramethylenediamine
Tris	Tris-hydroxymethyl-ammonuimethane
Tween 20	Polyoxyethylene-sorbitanmonolaurate
U	Units
V	Volt
v/v	Volume per volume
w/v	Weight per volume

SUMMARY

In this study a new *Dictyostelium* STE20-like protein kinase DST2 (*Dictyostelium* STE20-like kinase 2) was cloned and characterised. STE20 (Sterile 20) kinase was first identified in yeast as a pheromone-induced serine/threonine protein kinase that acts upstream of a MAP kinase cascade. Based on the domain structure, DST2 belongs to the GCK subfamily of STE20-like protein kinases, which include the mammalian STE20-like kinases (MST1/2/3), oxidant stress response kinase SOK-1, and DST1 in *Dictyostelium discoideum* which phosphorylates severin, a gelsolin-like F-actin fragmenting protein. DST2 was discovered by screening of the *D. discoideum* cDNA project database using DST1 as query. To confirm the existence of the DST2 gene and its expression, Southern, Northern and Western analyses of DST2 were carried out. It revealed that DST2 may have two copies in the *Dictyostelium* genome and that DST2 was expressed during all stages of *D. discoideum* development. *In vitro* kinase assays with bacterially expressed fusion protein of full length DST2 (aa461), the catalytic domain (aa287) and the regulatory domain (aa174) showed that autophosphorylation of DST2 occurs on the regulatory domain and phosphorylates severin in the presence of a Mn^{2+} or Mg^{2+}. Purified catalytic domain of PKA phosphorylated the regulatory domain of DST2 and caused an increase in the basal autophosphorylation activity of DST2, suggesting that PKA may be a potential upstream kinase of DST2 through the phosphorylation of its regulatory domain. To understand the function of the non-catalytic domain of DST2, three C-terminal truncation constructs (aa1-421, aa1-368 and aa1-326) were used in comparison to full length DST2 in *in vitro* kinase assays. Deletion of C-terminal regions revealed an inhibitory region amino acids 326-461 of DST2. Gel filtration chromatography showed that DST2 was eluted in a broad peak ranging from approximately 63 kDa to 400 kDa, suggesting that DST2 may exist *in vivo* as a monomer as well as a high molecular weight complex. The influence of phosphorylated and unphosphorylated severin on F-actin solutions was investigated using falling-ball viscometry and fluorescence spectroscopy. It turned out that phosphorylation by DST2 inhibits the F-actin fragmenting activity of severin, suggesting that DST2 may be directly involved in actin-cytoskeleton rearrangements.

Zusammenfassung

In der vorliegenden Studie wird die Klonierung und Charakterisierung einer neuen STE20-ähnlichen Proteinkinase aus *Dictyostelium discoideum* beschrieben. STE20-Kinasen wurden zuerst in Hefe gefunden und stellen eine Hauptgruppe der p21-aktivierten Proteinkinasen dar. Die Domänenstruktur der *Dictyostelium* STE20-ähnlichen Kinase DST2 zeigt, dass sie der GCK Untergruppe ("germinal center kinases") zuzuordnen ist. Typische Vertreter dieser Untergruppe sind die "mammalian STE20-like kinases" MST1/2/3, die durch oxidativen Stress induzierte humane Kinase SOK-1, und auch DST1, eine homologe Kinase aus *Dictyostelium*. DST2 wurde beim Durchsuchen der *D. discoideum* cDNA Datenbanken gefunden. Zunächst wurden im Rahmen dieser Doktorarbeit das entsprechende Gen mit Hilfe von Southern Analysen, sowie die Expression durch Northern und Western Experimente untersucht. Es stellte sich heraus, dass es im *Dictyostelium* Genom wohl zwei Kopien für DST2 gibt und dass das Protein in allen Stadien der *Dictyostelium* Entwicklung vorhanden ist. *In vitro* Kinase-Assays mit bakteriell exprimiertem Fusionsprotein (gesamte Kinase, katalytische oder regulatorische Domäne) zeigten, dass die Autophosphorylierung von DST2 in der regulatorischen Domäne stattfindet und dass die Phosphorylierung von Severin auch durch Mn^{2+} beeinflusst werden kann. DST2 ist *in vitro* außerdem ein Substrat der PKA, die DST2 in der regulatorischen Domäne phosphoryliert und dadurch die basale Aktivität der Autophosphorylierung erhöht. Dieser Befund lässt vermuten, dass PKA *in vivo* möglicherweise stromaufwärts der DST2 auf die Regulation dieses Signalweges Einfluss nimmt. Zum besseren Verständnis der regulatorischen Domäne in DST2 wurden C-terminal verkürzte Konstrukte hergestellt (As 1-421, As 1-368, As 1-326) und mit der Aktivität der kompletten DST2 in *in vitro* Assays verglichen. Es stellte sich heraus, dass der C-Terminus die katalytische Aktivität der Kinase inhibiert. In der Gelfiltration konnte kein eindeutiges Molekulargewicht der Kinase festgestellt werden, in der Regel wurde die Kinase in einem breiten Peak zwischen 400,000 - 63,000 eluiert. Diese Ergebnisse deuten darauf hin, dass DST2 *in vivo* sowohl als Monomer als auch als hochmolekularer Komplex vorliegen kann. Durch Viskosimetrie- und Fluoreszenzmessungen konnte nachgewiesen werden, dass Severin in seiner F-Aktin fragmentierenden Aktivität durch Phosphorylierung gehemmt wird. Dieses Ergebnis lässt vermuten, dass DST2 möglicherweise direkt am dynamischen Umbau des Aktin-Zytoskeletts beteiligt ist.

CHAPTER 1

Introduction

1.1 The cytoskeleton

The cytoskeleton is a complex network of protein filaments that extend throughout the cytoplasm. It is responsible for cell shape, cell motility, cell polarity, cytokinesis, intracellular transport, cytoplasmic streaming and muscle contraction. The diverse activities of the cytoskeleton depend on three types of protein filaments: actin filaments (about 6-8 nm in diameter), microtubules (about 25 nm in diameter) and intermediate filaments (about 10 nm in diameter). It is characteristic of these filamentous systems that they are built by reversible assembly of monomeric, evolutionarily highly conserved subunits, namely α- and β-tubulin in microtubules, the different proteins in intermediate filaments and actin in microfilaments (Sandoz *et al.*, 1988).

Intermediate filaments stabilize the cell against mechanical stress and structure the cytoplasm by establishing links to various binding partners (Housewart and Cleveland, 1998). However, no direct involvement of intermediate filaments in cellular motility has been reported to date. In contrast, actin filaments and microtubules interact with different proteins to generate different types of cellular motility. Microtubules are major organizers of the cell interior and are vitally involved in motility events such as chromosome migration during cell division (Valiron *et al*., 2001).

1. 2 Actin and actin binding proteins

The microfilament protein actin is not only the most abundant protein in many eukaryotic cells, but is also very highly conserved in evolution from human to amoeba. The 42 kDa globular actin monomer (G-actin) polymerizes into polar, helical filaments (F-actin). Actin filaments have a polar structure, with two structurally different ends - a fast growing (barbed or +) end and a slow growing (pointed or -) end (Wegner, 1976).

In vivo, actin polymerisation is a highly regulated process controlled both by ATP binding and hydrolysis, and by the action of a number of actin binding proteins that control the incorporation of actin monomers into existing filaments, the dynamic equilibrium between G- and F-actin and the three-dimensional organization of the filamentous network (Schleicher *et al*, 1995). For many actin-binding proteins it was shown that their activity is regulated *in vitro* by Ca^{2+}, phospholipids, phosphorylation or changes in pH. Based on their interaction with G- or F-actin, they are placed into different functional groups. One distinguishes between proteins that bind actin monomers, fragment and/or cap actin filaments, act as molecular motors, tether actin filaments to the membrane, or crosslink actin filaments (Eichinger *et al*., 1999).

The actin-based motility is driven by the assembly of actin filaments. Important regulatory proteins in the assembly of new actin filament networks are the Arp2/3 complex and the Wiscott-Aldrich Syndrome Protein (WASP). The Arp2/3 complex is a complex of seven proteins, including the actin-related proteins Arp2 and Arp3. It localizes to the leading edge of a variety of cells, binds to the sides of actin filaments and rapidly nucleates branches (Svitkina and Borisy, 1999; Bear *et al*., 2002). Members of the WASP and the related Scar (Supressor of cAMP receptor) family bind directly to the Arp2/3 complex and stimulate its ability to promote the nucleation of new actin filaments (Machesky and Insall, 1998; Seastone *et al*., 2001). F-actin fragmenting and/or capping proteins, that have also been identified as important regulators of cell motility, are described in the next section.

1.3 F-actin fragmenting proteins

Filament number and length are in part controlled by F-actin fragmenting proteins. At micromolar Ca^{2+} concentrations they sever actin filaments, which is usually followed by capping of the newly created barbed end. This leads to a rapid increase of short capped filaments together with a dramatic decrease in viscosity. For several members of this family it has been shown that uncapping is caused by polyphosphoinositides, particularly phosphatidylinositol 4,5-biphosphate (PIP_2) (Janmey *et al*., 1987; Eichinger and Schleicher, 1992). *In vivo* this could lead to free barbed ends ready for rapid elongation (Stossel, 1989).

The best characterized and most important members of this group are gelsolin and villin from vertebrates, severin from *Dictyostelium discoideum* and fragmin from *Physarum polycephalum* (Yin and Stossel, 1979; Bretscher and Weber, 1979; André *et al.*, 1988; Hasegawa *et al.*, 1980; Schleicher *et al.*, 1988). Gelsolin is characterized by two homologous halves, each with threefold repeated segments of approximately 15 kDa. The 15 kDa domain is crucial for classification and is so far present in all identified gelsolin-like proteins in three (severin, fragmin) to six copies (gelsolin, villin). Villin contains in addition a seventh carboxyterminal domain, the so-called headpiece (HP) which is responsible for its F-actin crosslinking activity (Fig. 1).

Severin is an abundant 40 kDa cytoplasmic protein that is expressed throughout *D. discoideum* development (Brown *et al*., 1982). Domain analysis showed that the severin domains have distinct functions. The first domain is responsible for the capping activity, whereas domains 2 and 3 contain binding sites for the sides of actin filaments and domain 1 together with at least part of domain 2 is needed for the F-actin fragmenting activity (Eichinger *et al*., 1991; Eichinger and Schleicher, 1992).

There is increasing evidence that actin fragmenting proteins might be targets in signalling cascades to the cytoskeleton. Gelsolin has been implicated in the phosphoinositide-mediated F-actin uncapping of human platelets following stimulation of thrombin receptors (Hartwig *et al*., 1995). Fibroblasts of gelsolin null mice have excessive actin stress fibers and migrate more slowly than wild type fibroblasts (Witke *et al*., 1995), while overexpression of gelsolin in NIH 3T3 fibroblasts leads to an increase in motility (Cunningham *et al*., 1991). Studies in humans with hereditary gelsolin-related amyloidosis (AGel amyloidosis), showed that the gelsolin gene defect causes altered gelsolin-mediated cellular mechanisms, which may contribute to a bleeding tendency in AGel amyloidosis patients (Kiuru *et al*., 2000). In addition to Ca^{2+} and poly-phosphoinositides, phosphorylation seems to play an important role in regulating proteins from this family as well (see 1.5 last paragraph).

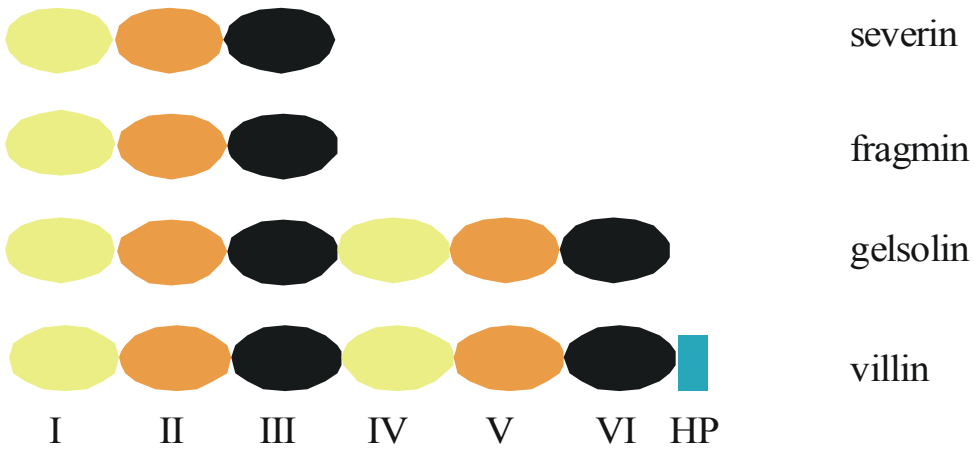


Fig. 1: Domain organisation of severin, fragmin, gelsolin and villin.

The domain organisation of *Dictyostelium* severin, *Physarum* fragmin and vertebrate gelsolin and villin is schematically depicted. Severin and fragmin have a three-fold repeated domain structure. Gelsolin contains two homologous halves, each with threefold repeated segments. Villin contains an additional C-terminal domain the so-called headpiece domain (HP). Roman numerals indicate the domain number.

1.4 Actin-binding proteins as substrates for protein kinases

To understand the signalling pathways that link external signals to the regulation of the actin cytoskeleton, potential upstream kinases for actin-binding proteins need to be characterized. *In vitro* and *in vivo* phosphorylation has been shown for a number of cytoskeletal proteins and for some of these it was shown that phosphorylation regulates their activity. A prominent example is cofilin, a G-actin binding and F-actin fragmenting protein, which has been identified as substrate for LIM-kinase1 (Arber *et al*., 1998). LIM-kinase1 (LIMK1), a serine/threonine kinase, phosphorylates cofilin at Ser 3, both *in vitro* and *in vivo*. Phosphorylation at Ser 3 inhibits the actin binding and depolymerisation activity of cofilin. An active form of Rac increases the activity of LIMK-1, suggesting that LIMK-1 and cofilin might be downstream effectors of the Rac-mediated formation of lamellipodia. LIMK-1 might therefore be a key component of a signal transduction cascade that connects extracellular stimuli to changes in cytoskeletal structure (Yang *et al*., 1998). Spatial and temporal aspects of this regulatory cascade still need to be clarified.

1.5 STE20-like kinases

The MAPK (Mitogen Activated Protein Kinase) system is an evolutionarily highly conserved intracellular signalling cascade and plays an essential role in many cellular processes, such as growth, differentiation and stress-related response. Its core comprises a module of three kinases consisting of a MAPKKK (MAPK kinase kinase), MAPKK (MAPK kinase) and MAPK. STE20 (Sterile 20) is a pheromone-induced yeast serine/threonine protein kinase that acts upstream of a MAP kinase cascade (Wu *et al*., 1995; Leberer *et al*., 1992). In response to activated Cdc42, it activates the MAPK cascade that includes STE11 (MAPKKK), STE7 (MAPKK) and FUS3/KSS1 (MAPK) (Herskowitz *et al*., 1995). STE20-related protein kinases have been identified in various eukaryotes, and have a highly conserved catalytic domain in common with yeast STE20. Based on their structure and regulation, they can be divided into two subfamilies.

The first group, including yeast STE20 and its mammalian homologues, the PAKs (p21 activated kinases), have a kinase domain at the carboxy terminus and a putative regulatory domain at the amino terminus, which contains a binding site for Rac1 and Cdc42 (Manser *et al*., 1994). PAKs are regulated *in vivo* and *in vitro* by the small GTP binding proteins Rac1 and Cdc42 and by phospholipids. PAKs specially regulate the JNK pathway and are involved in regulating some of the diverse cytoskeletal changes induced by Rac and Cdc42 (Bagrodia *et al*., 1995; Kyriakis *et al*., 1996; Benner *et al*., 1995; Yu *et al*., 1998; Bokoch *et al*., 1998). They have been shown to be required for processes including neurite formation and axonal guidance, development of cell polarity and motile responses (Daniels and Bokoch, 1999).

The second group, the so-called GCK (Germinal Center Kinase) subfamily, has a catalytic domain at the N-terminus and a putative regulatory domain at the C-terminus and lacks a recognizable GTPase binding site (Sells and Chernoff, 1997). The GCKs subfamily can be further divided into two groups based on their structure and properties. Group I GCKs are closely related to GCK. This group consists of GCK, GCKR (GCK-related), GLK (GCK-like), HPK1 (Hematopoietic Progenitor Kinase-1) and NIK (Nck-Interacting Kinase) (Katz *et al*., 1994; Kiefer *et al*., 1996; Hu *et al*., 1996; Su *et al*., 1997; Diener *et al*., 1997; Shi *et al*., 1997; Fu *et al*., 1999). Mammalian SOK-1 (STE20-like oxidant stress-activated kinase1), MST1, 2 and 3 (mammalian STE20-like kinase 1, 2 and 3) and LOK (Lymphocyte-Oriented Kinase), *Dictyostelium* DST1 and yeast Sps1 belong to the group II GCKs (Pombo *et al*., 1996; Creasy and Chernoff, 1995a; Creasy and Chernoff, 1995b; Schinkmann and Blenis, 1997; Friesen *et al*., 1994; Eichinger *et al*., 1998).

These enzymes are less well understood. Although group II GCKs share homologous sequence, with the catalytic domain of group I GCKs, their C-terminal regulatory domains differ significantly from those of the group I kinases (Eichinger *et al*., 1998; Friesen *et al*., 1994; Schweitzer and Philippsen, 1991).

Several members of the GCK subfamily are responsive to cellular stress. Sps1p has been shown to become activated in response to nutrient deprivation (Friesen *et al*., 1994). Human Krs-1 and Krs-2, which are identical with MST1 and MST2, are activated upon treatment of cells with staurosporine, okadaic acid, high concentrations of sodium arsenite, and extreme heat shock at 55°C (Taylor *et al*., 1996; Creasy and Chernoff, 1995a; Creasy and Chernoff, 1995b). Furthermore the activity human SOK-1 was shown to be induced several fold by oxidant stress. It most likely controls a novel stress response pathway since it is not involved in already defined MAPK cascades (Pombo *et al*., 1996). This lead to the assumption that members from the GCK subfamily are important for the response of eukaryotic cells to environmental stresses. Recent results suggest that some members of the GCK subfamily might also be involved in the regulation of the remodelling of the actin cytoskeleton via F-actin fragmenting proteins. Severin was identified as a substrate for DST1 *in vitro*. Phosphorylation of severin was strongly reduced in the presence of Ca^{2+} (Eichinger *et al*.,1998). Though there is no direct proof, several pieces of evidence suggest that severin is also an *in vivo* substrate for DST1 (unpublished results). Furthermore, TNIK was shown to phosphorylate gelsolin. Overexpression of wild type TNIK in NIH3T3 and Hela cells lead to morphological changes of the cells and resulted in the disruption of F-actin structures and inhibition of cell spreading (Fu *et al*., 1999).

1.6 *Dictyostelium discoideum*

Cellular slime molds were first discovered by O. Brefeld in 1869, but the modern era began with the discovery of the new species *Dictyostelium discoideum* (Raper, 1935). *D. discoideum* has two alternative life cycles: sexual and asexual. The asexual cycle is easier to produce in the laboratory and is the one used for almost all experimental studies. *D. discoideum* feeds on bacteria as separate amoebae and upon starvation enters a social, multicellular stage which is mediated by chemotaxis. Chemotactically aggregated cells form a multicellular structure which undergoes morphogenesis and cell-type differentiation into spore and stalk cells.

Development culminates in the generation of fruiting bodies, which contain resistant spores, and can give rise to individual amoebae (Loomis *et al.*, 1996). The life cycle of *Dictyostelium* is summarized in Fig. 2.

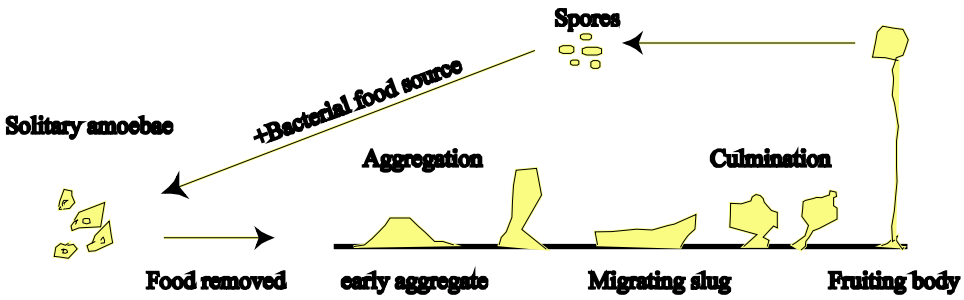


Fig. 2: The life cycle of *Dictyostelium discoideum*

Spores germinate to give rise to individual amoebas, which feed on bacteria as a food source, and the vegetative cells divide and multiply. Upon depletion of the food source, the cells chemotatically aggregate to form a cell mass that elongates to form the "first finger", which then either migrates as a slug or directly enters the culmination stage leading to the formation of a fruiting body consisting of the terminally differentiated stalks cells and the spores which can initiate another round of the cycle.

The advantages of studying *Dictyostelium* as a model system is that a large amount of genetically identical cells can be easily cultivated for biochemical analysis. A small genome of about 34Mb of DNA facilitates the molecular studies of genes involved in growth and development. Furthermore, a wide variety of improved genetic approaches are available to generate mutants for studying the cytoskeleton of *Dictyostelium*. These include targeted gene disruption and gene replacement by homologous recombination (Manstein *et al*., 1989), antisense-mediated gene inactivation (Knecht and Loomis, 1987; Liu *et al*., 1992) and the transposon tagging like mutagenesis by restriction enzyme-mediated integration (REMI) of DNA (Kuspa and Loomis, 1992; Karakesisoglou *et al.*, 1999). *Dictyostelium* is also a good model system for the analysis of the actin cytoskeleton and has contributed to the general understanding of the structure and function of cytoskeletal proteins (Schleicher and Noegel, 1992; Noegel and Luna, 1995). In summary, *Dictyostelium* is an excellent experimental system for studies of the role of the actin cytoskeleton in cell motility, chemotaxis, signal transduction, development and differentiation.

1.7 Goals of the project

The goal of this project was to discover and characterise new protein kinases homologous to DST1 in *D. discoideum.* Previous work showed that DST1 phosphorylates the F-actin fragmenting protein severin (Eichinger *et al*.,1998). These results suggested that DST1 might play a regulating role in the rearrangement of the actin cytoskeleton. DST2 (*Dictyostelium* STE20-like kinase 2) was discovered by screening the sequences of the *D. discoideum* data bases. DST2 is a serine/threonine protein kinase and highly homologous to DST1 (69% similarity in the kinase domain).

The further aims of this work were to biochemically characterise DST2, to understand its function in terms of a possible regulatory role in the remodelling of the actin cytoskeleton, and to investigate in which signalling pathways DST2 might be involved.

To address these questions, the following approaches were taken :

1) DST2 was generated as active recombinant protein kinase and used for biochemical characterisation and to test various potential substrates.
2) A polyclonal antiserum was raised against recombinant DST2 and used in cell biological and biochemical studies in *D. discoideum.*
3) Various C-terminal truncated DST2 constructs were generated and characterised in biochemical assays to unravel functionally important regions in the C-terminal domain.
4) Recombinant DST2 was used to study the impact of phosphorylation on severin activity in viscometry- and fluorescence spectroscopy assays.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Enzymes for molecular biology

Calf intestine alkaline phosphatase	Roche
DNA polymerase 1	Roche
Lysozyme	Sigma
Restriction enzymes	Amersham, Boehringer, Eurogentec, Gibco-BRL, New England Biolabs, Promega
RNase A	Sigma
T4 DNA ligase	Gibco-BRL, Promega
Taq polymerase	Amersham
Pfu Turbo polymerase	Stratagene

2.1.2 Antibodies

Anti-Actin (mAb Act1)	(Simpson *et al*., 1984)
Goat anti-mouse IgG antibody, coupled with peroxidase	Dianova
Goat anti-mouse IgG antibody, Conjugated with Cy3 or FITC	Dianova

2.1.3 Protease inhibitors

Benzamidine	Sigma
PEFA-block	Roth
Phenylmethylsulfonylfluoride (PMSF)	Serva
Protease inhibitor cocktail (p2714)	Sigma

2.1.4 Antibiotics

Ampicillin	Roth
Blasticidin S	ICN Biomedicals
Geneticin (G418)	ICN Biomedicals
Hygromycin B	Cabiochem
Kanamycin	Sigma
Nalidixic acid	Sigma
Penicillin/Streptomycin	Sigma

2.1.5 Chemical reagents

Unless otherwise stated, chemicals were obtained from Fluka, Merck, Pharmacia, Roth, Serva or Sigma and have the purity grade of "p.a".

Agarose (SeaKem Me)	FMC Bioproducts
Bacto-agar, -peptone, -tryptone	Difco
Chloroform p.a	Riedel de Haen
DE52 (Diethylaminoethyl-cellulose)	Whatman
Hydroxylapatite	Bio-Rad
IPTG (Isopropyl-β-D-thiogalactose-pyranosid)	Gerbu
Oligonucleotides	MWG-biotech
Peptone	Oxoid
Phenol	Appligene
Phosphocellulose (P11)	Whatman

Proteose peptone	Oxoid
Triton X-100	Pierce
Yeast extract	Oxoid

2.1.6 Media

All media and buffers were made with deionized water and sterilized by autoclaving at 120ºC for 20 min. Antibiotics were added to media when cooled to about 50ºC.

2.1.6.1 Media for *D. discoideum* culture

AX medium (pH 6.7)

14.3 g	peptone
7.15 g	yeast extract
50 mM	glucose
3.5 mM	Na_2HPO_4
3.5 mM	KH_2PO_4

SM agar plates (pH 6.5)

9 g	agar
10 g	peptone
50 mM	glucose
1 g	yeast extract
4 mM	$MgSO_4$
16 mM	KH_2PO_4
5.7 mM	K_2HPO_4

-Both media were filled up to 1 litre with dH_2O.

Soerensen Phosphate buffer (pH 6.0)

14.6 mM	KH_2PO_4
2 mM	Na_2HPO_4

Salt solution

10 mM	NaCl
10 mM	KCl
2.7 mM	$CaCl_2$

HL-5 medium

10 g	yeast extract
50 mM	glucose
8.5 mM	KH_2PO_4
1.25 mM	Na_2HPO_4

-filled up to 2 L with dH_2O

Phosphate agar plates (pH 6.0)

15 g	Bacto agar

- filled up to 1 L with Soerensen buffer

2.1.6.2 Medium for *E. coli* culture

LB-medium (pH 7.4)

10 g Bacto-tryptone

5 g yeast extract

86 mM NaCl

-pH adjusted with NaOH and medium filled up to 1 L with dH_2O.

For LB-agar plates, 1.5% (W/V) agar added into the medium and selection was provided by introducing 50 mg/l ampicillin and/or 25 mg/l kanamycin

2.1.7 Buffers and other solutions

Those not shown here will be described in their corresponding sections under Methods.

100x Denhardt's reagent

2%	Ficoll 400
2%	polyvinylpyrrolidon
2%	Bovin serum albumin

TE buffer (pH 8.0)

10 mM	Tris/HCl
1 mM	EDTA

-autoclave

10 x NCP buffer (pH 8.0)

100 mM	Tris/HCl
1.5 M	NaCl
5 ml	Tween 20
0.02%	NaN_3

-Filled up to 1 L with dH_2O

10 x TBE buffer (pH 8.3)

890 mM	Tris
890 mM	Boric acid
20 mM	EDTA

-autoclave

PBS (pH 7.2)

70 mM	Na_2HPO_4
150 mM	NaCl
30 mM	KH_2PO_4
2.7 mM	KCl

-autoclave

Tris-Phenol (pH 8.0)

-1 vol. melted phenol was equilibrated with 1 vol. 1 M tris/HCl, pH 8.0

2.1.8 Equipment

Axiophot microscope	Zeiss
CCD camera (C5985-10)	Hamamatsu
Conductivity meter (LF 537)	WTW
Confocal laser scanning microcope	Leica
Diavert inverse microscope	Leica
Digital color video CCD camera (TK-C1380)	JVC
Dounce homogenizer	Braun
Eagle Eye II	Stratagene
Electroporation apparatus	BioRad
Fluorescence spectrophotometer (Aminco Bowman)	Sopra
FPLC device (BioLogic)	BioRad
Nuclepore filter	Costar
Parr bomb	Parr Instrument Company
PCR thermal cycler	Biometra
PH meter	Knick
Protein fraction collector	Pharmacia
Rotary shaker	GFL
Semi-dry protein transfer Trans-Blot SD	BioRad
SMART system	Pharmacia
Spectrophotometer	Pharmacia
Speed-Vac concentrator	Bachhofer
Stereomicroscope (MZ12)	Leica
Ultrafiltration centricon	Amicon
Vortex	Bender & Hobein
Water baths	GFL
Weighing machines	Sartorius
X-ray film developing machine (Curix 60)	AGFA

2.1.9 Other materials

2.2 ml sterile tubes for freezing of cells	Nunc
3 MM filter paper	Whatman
4-well borosilicate glass chamber slides	Nunc
24-well plates	Costar
Dialysis membrane	Biomol
Eppendorf tubes (0.1 ml, 0.5 ml, 1.5 ml)	Eppendorf
Falcon centrifuge tubes (15ml, 30ml)	Falcon
Nitrocellulose membranes (BA85)	Schleicher & Schuell
Petri dishes	Greiner
Polaroid film (667)	Polaroid
Polyallomer ultracentrifuge tubes 1.5 ml	Beckman
Quartz cuvettes	Hellma
Sterile filters (0.22 μm, 0.45 μm)	Millipore
Tissue culture flasks	Nunc
X-ray films (X-Omat)	Kodak

2.1.10 Centrifuges and rotors

Centrifuges

J2-21 M/E	Beckman
J6-HC	Beckman
G6-SKR	Beckman
Optima LE-80K ultracentrifuge	Beckman
Optima TL 100 ultracentrifuge	Beckman
Table-top centrifuge (5415)	Eppendorf

Rotors

JA 14, JA 20, JS-4.2, Ti45, Ti 70, TLA 100.3	Beckman

2.1.11 Computer programmes

Windows NT	
Bilddatenbank system	Leica
Sigma Plot 2.01	Jandel scientific
Winword 7.0	Microsoft
Macintosh	
Illustrator 8.0	Adobe
NIH Image 1.60	National Institutes of Health
Photoshop 5.0	Adobe
UNIX	
UWGCG package program (University of Wisconsin Genetics Computer Group)	

2.2 Vectors and Strains

2.2.1 Vectors

The following vectors were used: pMalC_2-vector (New England Biolabs), PQE30 (Qiagen).

2.2.2 Bacterial strains

The following *Escherichia coli* strains were used: XL-1 Blue for cloning (Sambrook *et al*., 1989), BL21 and M15 for protein expression.

2.2.3 Cultivation of *E. coli*

Bacteria were cultivated according to standard methods (Sambrook. *et al*., 1989) on agar plates or in liquid culture (240rpm). The cultivation temperature was 37°C. For long-term storage, 400 µl of bacterial culture were mixed with an equal amount of glycerol and stored at –80°C.

2.2.4 *D. discoideum* strains

The wild type *D. discoideum* strain AX2, which can be cultivated under axenic conditions, was used for this study.

2.2.5 Cultivation of *D. discoideum*

2.2.5.1 Growth in liquid medium (Claviez *et al*., 1982)

From spores or bacterial lawns on SM-agar plates, the wild type AX strain was inoculated into AX or HL-5 medium containing the antibiotics streptomycin sulfate (400 μg/ml) in order to *K. aerogenes*. The generation time at 21°C and 150 rpm is about 10 hours. For large-scale generation of cells for protein purification, cells cultivated in 4 x 2.5 l cultures up to a density between 5 x 10^6 and 1.2 x 10^7 cells/ml were harvested after centrifugation giving normally a yield of about 100g wet weight. As for cell biological studies, cells were allowed to grow up to a maximum density of 5 x 10^6 cells/ml to avoid the stationary phase.

2.2.5.2 Growth on agar plates

Isolation of transformant clones was carried out on SM agar plates. 100 μl of a suspension of *K. aerogenes* in salt solution were placed on each agar plate, spread evenly together with the *Dictyostelium* cells, and the plates were kept at 21°C for about 2 days. The doubling time is around 3-4 h and the *Dictyostelium* colonies appeared as round clearings on the bacteria lawn.

2.2.5.3 Preservation of spores

Cells from the axenic culture were harvested by centrifugation at 300 g for 10 min, washed once with cold Soerensen buffer and resuspended at a cell density of 2 x 10^8 cells/ ml. 500 μl of the cell suspension was spread out per phosphate agar plate, and the cells were able to develop into fruiting bodies within 2-3 days. The spores were then harvested by knocking onto the lid of the petri dish and taken up in 10 ml of cold Soerensen buffer. 1 ml aliquots were dispensed into Nunc tubes (2.2 ml), shock-frozen in liquid nitrogen and stored at -70°C.

For inoculation of spores, an aliquot was thawed at room temperature and cultured in AX medium, whereby after 3 days at 21ºC and 150 rpm, a cell density of about 5 x 10^6 cells/ml was reached.

2.2.5.4 Freezing of *Dictyostelium* cells

For the preservation of *Dictyostelium* cells, axenic cultures were harvested and resuspended at a cell density of about 5 x 10^7 cells/ml in ice-cold freezing medium (AX or HL-5 medium +1% (v/v) penicillin–streptomycin solution +5% (v/v) DMSO), and distributed as 1 ml aliquots into Nunc tubes (2.2 ml) pre-cooled on ice. The aliquots were then placed into the wells of a pre-cooled (4ºC) brass block (10 x 9 x 5 cm) and kept at -70ºC for a period of at least 15h and subsequently stored at -70ºC. To recover the cells, a frozen vial was thawed rapidly under cold running water, the cells washed once with cold AX medium, and then cultivated in AX medium in petri dishes or 24-wells plates. For the transformant clones, growth medium was changed after 24h and replaced with medium containing suitable antibiotics for selection.

2.2.5.5 Development of *D. discoideum*

Wild-type *Dictyostelium* (AX2 cells) were cultivated on SM plates at 21°C for 24 hours. Cells were harvested from different stages of development of *Dictyostelium* and used for Northern and Western analysis.

2.3 DNA methods

2.3.1 Agarose gel electrophoresis

The separation of DNA fragments according to length was done in gels with 1% agarose in TBE buffer. For the detection of DNA fragments, 0.05 µg/ml ethidium bromide was added to the liquid agarose. Gels were run with 50-200 V. Probes were incubated with 1/6 (v/v) sample dye before loading. After separation, the fragments were detected with a UV illuminator and documented with the Eagle Eye CCD camera system (Stratagene, Heidelberg).

DNA sample buffer
40% Sucrose
0.5% SDS
0.25% Bromophenol blue
-taken up in TE buffer

2.3.2 DNA extraction from agarose gels

DNA bands were cut out of the gel with a scalpel and transferred into a sterile micro tube. The isolation procedure was performed with the DNA extraction kit (Qiagen) according to the manufacturer's manual.

2.3.3 Determination of DNA concentration

DNA concentration in agarose gels was estimated by comparing the intensity of the band with the bands of the molecular weight marker. Alternatively, the OD_{260} was measured (50 µg/ml of DNA have an OD_{260} of 1, Sambrook *et al*., 1989).

2.3.4 Preparation of plasmid DNA

2.3.4.1 Isolation of plasmid DNA by the method of Homes and Quigley (1981)

An inoculum of bacteria was taken from a streak culture and suspended in 200µl of STET/lysozyme buffer. Alternatively, 1 ml of bacteria from a fresh overnight shaking culture was harvested using a table-top centrifuge (5000 rpm, 1 min, RT), the supernatant discarded and the pellet resuspended in STET buffer. The suspension was boiled in a water bath for 1 min, centrifuged (14,000 rpm, 10 min, RT), and the insoluble cell debris removed using a sterile toothpick. The nucleic acids in the supernatant were precipitated with 200 µl of isopropanol for 5 min at RT and sedimented using a table-top centrifuge (14,000 rpm, 30 min, RT). The DNA pellet was then washed with 70% ethanol, vacuum-dried with a Speed-vac concentrator, and dissolved in 30 µl of TE buffer.

STET buffer (pH 8.0)

50 mM	Tris/HCl
50 mM	EDTA
5%	Triton-X-100
8%	Sucrose
1 mg/ml	lysozyme (added prior to usage)

2.3.4.2 Isolation of plasmid DNA by the method of Qiagen

Large-scale preparation of plasmid DNA from *E. coli* was carried out with the Midi-kit purchased from the Qiagen company. Bacteria were cultivated in 150 ml of LB medium containing 50 μg/ml ampicillin overnight at 37°C with agitation. The cells were harvested by centrifugation (4,000 g, 5 min, 4° C) and resuspended in 10 ml of buffer P1. For cell lysis, 10 ml of buffer P2 was introduced and the resultant mixture was gently mixed by inverting the tube several times. After incubation for 5 min at RT, 10 ml of chilled buffer P3 was added, the suspension mixed gently by inverting tube, and then incubated on ice for 15 min. Following centrifugation (35,000 g, 30 min, 4° C), the sediment of proteins and cell debris was discarded, while the supernatant containing the plasmid DNA was centrifuged for a further period of 15 min. The clear supernatant was passed through a Qiagen tip 100 column previously equilibrated with 5 ml of buffer QBT. After washing twice with 10 ml each of buffer QC, the plasmid DNA was eluted with 5 ml of buffer QF, precipitated with 0.7 vol. of isopropanol, spun down (30,000 g, 30 min, 4°C), and the DNA pellet washed with 70% ice-cold ethanol, air-dried and dissolved in 200 μl of TE buffer. The DNA concentration was determined using a spectrophotometer whereby an OD_{260} of 1.0 corresponds to 50 μg of DNA. The ratio of OD_{260}/OD_{280} should be between 1.8 and 2.0.

2.3.4.3 Phenol Extraction and Precipitation of DNA

To separate the DNA from contaminating proteins, the DNA solution was mixed with 50% (v/v) phenol and 50% (v/v) chloroform/isoamylalcohol (24:1). The mixture was heavily vortexed and centrifuged at 13,000 rpm at room temperature for 5 min. The upper phase was collected and the DNA was precipitated with ethanol. For ethanol precipitation, 10% (v/v) of a 3 M Na-acetate solution of pH 5.2 as well as 2.5 volumes of ethanol were added to the DNA solution. The sample was then incubated at –80ºC for 1 hour. After a centrifugation of 30 min at 13,000 rpm and 4ºC, the pellet was washed with 70% ethanol, centrifuged again for 15 min under the previous conditions and dried in a Speed Vac. The dried pellet was resuspended in a suitable volume of H_2O.

2.3.5 DNA cleavage with restriction enzymes

The cleavage was performed in a volume of 50 µl. The buffer suitable for the restriction enzyme(s) was added and the volume adjusted with H_2O. After addition of the enzyme(s), the tube was incubated at the appropriate temperature for at least 1h. The digest was analysed on an agarose gel.

2.3.6 Ligation of DNA into a plasmid vector

To avoid re-ligation and concatamer formation of a cut vector, the vector was dephosphorylated with alkaline phosphatase (CIP, Calf Intestinal Phosphatase). The reaction was performed according to manufacturer's instructions (New England Biolabs). The vector was purified on a gel and incubated with the corresponding insert. The reaction volume was set to 10 µl. 50 ng of vector were ligated with a threefold molar excess of insert. Ligases of various suppliers were used (Gibco, NEB) together with the supplied buffers. After the ligation, the DNA was extracted with phenol, precipitated with ethanol and resuspended in 5-10 µl of H_20.

2.3.7 Preparation of electroporation competent cells

1 liter LB was inoculated with 10 ml of an *E. coli* overnight culture and grown to an OD_{600} of 0.6 at 37°C under vigorous shaking. All flasks and solutions subsequently used were sterilized and cooled to 4°C. Proper cooling was essential for obtaining competent cells of a good quality. Cells were spun down and resuspended in 1 l of H_20. After another centrifugation, the cells were resuspended in 500 ml of H_2O, pelleted again, washed with 20 ml of 10% glycerol and finally resuspended in 3 ml of 10% glycerol. The cells were stored at -80°C in 50 µl aliquots.

2.3.8 Electroporation of *E. coli*

For transformation, electroporation competent *E. coli* cells were thawed on ice. 50 µl of cells were mixed with 1-5 µl of DNA or the ligation mixture resolubilized in dH_2O, and placed in a pre-chilled electroporation cuvette (Eurogentec; 2 mm gap between electrodes). After a pulse of 2.5 kV, 200 Ω and 25 µF, 1 ml of SOC medium (Sambrook *et al.*, 1989) was immediately added and the cells regenerated at 37°C for 45 min with agitation. 1, 10 and 100 µl of cells were plated out on LB agar plates containing 50 µg/ml of ampicillin or 25 µg/ml of kanamycin and incubated overnight at 37°C.

SOC medium

2%	Bacto-tryptone
0.5%	Yeast extract
10 mM	NaCl
2.5 mM	KCl
10 mM	$MgCl_2$
10 mM	$MgSO_4$
20 mM	Glucose

2.3.9 Screening for positive *E.coli* transformants

To isolate *E. coli* colonies carrying the desired DNA fragment, colonies were selected and mini preparations of plasmid DNA were performed followed by restriction analysis to determine the correct orientation of the DNA insert. Finally the authenticity of the DNA sequence was verified by sequencing (Toplab, Martinsried).

2.3.10 *E. coli* permanent cultures

Important transformants were preserved as permanent cultures. An inoculum of bacteria was resuspended in 1 ml of LB medium containing 7% of DMSO. The cells were shock frozen in liquid nitrogen and stored at -70°C.

2.3.11 Transformation of *D. discoideum*

Recombinant gene expression in *D. discoideum* was regulated by a plasmid harbouring the *Dictyostelium* actin 15 promoter (A15P) and actin 8 terminator (A8T) sequences. The presence of an appropriate antibiotic resistance cassette on the expression plasmid allowed for selection of transformants with geneticin or blasticidin.

For transformation, *Dictyostelium* cells cultivated to a density of 2-3 x 10^6 cells/ml were harvested by centrifugation (300 g, 5 min, 4°C), washed once with cold Soerensen buffer, followed by washing twice with ice-cold electroporation buffer and the cells finally resuspended in chilled electroporation buffer at a density of 1 x 10^8 cells/ml. 500 µl of the cell suspension were mixed with 15-30 µg of the desired plasmid DNA in a pre-chilled electroporation cuvette (4 mm gap between electrodes). After a pulse at 1.0 kV and 3 µF with the aid of an electroporation device (Gene Pulser, Biorad), the cells were immediately transferred to a petri dish and incubated at RT for 10 min, after which $CaCl_2$ and $MgCl_2$ were added to final concentration of 1 mM each and the cells incubated for an additional 15 min at RT with gentle agitation. Finally, the cells were diluted with HL-5 medium to a density of 1 x 10^6 cells/ml and allowed to recover at 21°C for 24 h before selection pressure was added.

Electroporation buffer (pH 6.1)

50 mM sucrose

10 mM KH_2PO_4

-pH was adjusted with KOH solution, and the buffer sterilized by filtration

2.3.12 Polymerase chain reaction (PCR)

The amplification of DNA fragments was carried out by "polymerase chain reaction". For PCR (colony screening), *Taq* polymerase (Boehringer) and Pfu Turbo polymerase (Boehringer) were used. The reactions contained 2 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM 5'- and 3'- primer and 1/250 vol of Taq-polymerase. cDNA, genomic DNA and plasmid DNA were used as templates. Prior to amplification, the DNA was denaturated for 5 min at 95ºC. The denaturation was followed by 25 amplification cycles: 95ºC, 1 min; 50-65ºC (depending on the annealing temperature of the primers used), 1 min; 72ºC 1 min-3 min (depending on the length of the fragment to be amplified). The last elongation step was at 72ºC, for 3 min. Based on the number of guanine and pyrimidine nucleotides, the annealing temperature (Tm) of a oligonucleotide primer could be calculated according to the formula of Suggs *et al*. (1981) : 4(G+C) +2(A+T) – 10 = Tm (ºC). Preparative PCR was carried out with the Expand high fidelity PCR system from Boehringer Mannheim. The reactions were carried out according to the supplier's instructions.

2.3.13 Purification of PCR products

For cloning purposes, the PCR products were purified using the QIAquick PCR purification kit (Qiagen) following the manufacturer's protocol. Buffers used were provided by the kit and all centrifugation steps were done at 14,000 rpm at RT using a table-top microcentrifuge. 5 vol. PB buffer were added to 1 vol. of the PCR reaction and mixed. The sample was applied to a QIAquick spin column and centrifuged for 1 min to bind DNA to the column while the flow-through collected in 2 ml collection tube was discarded. DNA was washed with 0.75 ml of PE buffer by centrifuging for 1 min. Residual ethanol was removed by centrifuging for an extra 1 min. The spin column was then placed in a clean 1.5 ml eppendorf tube and the DNA eluted by the addition of 50 μl of dH_2O to the column and centrifuging for 1 min. The purified DNA was subsequently used for restriction digest for 2-6 h and finally purified from agarose gel with the aid of the QIAquick gel extraction kit from Qiagen.

2.3.14 Oligonucleotides (Primers)

DST2-Nt	5'- CGC GGA TCC ATG TCA ACG CTC AAT GTA CC-3'
DST2-Ct	5'-GCG CTG CAG CTA CTA CTT TGA TTT CTT TTC ATC-3'
DST2-Ntreg	5'-CGC GGA TCC GAA CAA GAT ATA ATC ATC AAT-3'
DST2-CtCat	5'-GCG CTG CAG ATC TAT TAA TGG TAC CAA GAG TG-3'
DST2-Ct326	5'-GCG CTG CAG CTA CTA TCC TCT ATT ATC ATC AGA ATC-3'
DST2-Ct368	5'-GCG CTG CAG CTA CTA ATT AAA TAC AAC TGT ATC ATA AG-3'
DST2-Ct421	5'-GCG CTG CAG CTA CTA CTC TAA GGA ATA ACT AGA G-3'
Av09uni1	5'-CGC GGA TCC AGT TAG AAA GAT TCA TAG AG- 3'
Av09rev1	5'-CGC GGA TCC AAT TTC TTG AAT GAC TTC-3'
Av09uni2	5'-GCA AAA TTC AGG TGG TGA AG-3'
Av09rev2	5'-CGC GGA TCC ATA AGA ATT CTT ATT ATC AG-3'

DST2-Nt/DST2-Ct: used for the complete construct of DST2

DST2-Nt/DST2-Ctcat: used for the catalytic domain of DST2

DST2-NtReg/DST2-Ct: used for the regulatory domain of DST2

DST2-Nt/DST2-Ct326: used for the c-terminal truncation construct of DST2 (1-326)

DST2-Nt/DST2-Ct368: used for the c-terminal truncation construct of DST2 (1-368)

DST2-Nt/DST2-Ct421: used for the c-terminal truncation construct of DST2 (1-421)

Av09uni1: used for sequencing

Av09rev1: used for sequencing

Av09uni2: used for sequencing

Av09rev2: used for sequencing

2.3.15 Southern Blotting

Dictyostelium genomic DNA was digested with various restriction enzymes and separated on 0.7% agarose gel (30 V, for 24 h). DNA was denatured in 0.5 M NaOH for 20 min and the gel was rinsed briefly with H_2O. The gel was submerged in neutralization solution (1 M Tris, pH 7.5 for 20 min) and equilibrated with 20 x SSC buffer for 20 min. DNA was blotted from the gel by capillary transfer to the membrane (nitrocellulose filter; Schleicher & Schuell) using 20 x SSC buffer. The membrane was rinsed with 6 x SSC buffer, baked at 80°C for 1.5 h, rinsed with hybridization buffer and hybridized with ^{32}P-labelled sample overnight.

The membrane was rinsed 3 times with 2 x SSC/ 0.1% SDS- buffer at 37°C for 10 min and rinsed again with wash buffer at 37°C for 1 h.

20 X SSC (pH 7.5)
3 M NaCl
0.4% Na-citrate

2.3.16 Northern blotting

For isolation of RNA, cells were lysed with 1% SDS in the presence of 0.1% diethylpyrocarbonate. The RNA was further purified by several phenol/chloroform extractions. RNA was separated on 0.8% agarose gels in the presence of 6% formaldehyde, transferred to nitrocellulose filters (Schleicher & Schuell) and probed with nick-translated DNA in 50% formamide, 2 x SSC, 1% sarkosyl, 4 mM EDTA, 0.1% sodium dedocyl sulfate, 4x Denhardt's solution and 0.12M phosphate buffer, pH 6.8, at 37°C for 16 to 18 h.

2.4 Analysis of DST2 with biochemical methods

2.4.1 SDS-Polyacrylamide gel electrophorosis (SDS-PAGE)

Protein mixtures were separated by discontinuous SDS-PAGE (Laemmli, 1970). For this purpose, 10-15% resolving gels with 3% stacking gels were used (7.5 x 10 x 0.05 cm). The stacking gel deposits the polypeptides to the same starting level at the surface of the resolving gel, and subsequently the SDS-polypeptides complexes are separated in the resolving gel according to size under uniform voltage and pH. Prior to SDS-PAGE, 1/3 vol. 3 x SDS gel loading buffer was added to the protein samples to be separated and boiled for 3 min. Electrophoresis was carried out at a constant voltage of 150 V, after which the gel could be stained with Coomassie Blue dye and destained for direct observation of the protein bands, or protein from the gel could be blotted onto nitrocellulose membranes and detected indirectly via antibodies. As standard, a mixture of proteins of defined molecular masses was electrophoresed.

Stacking gel

125 mM	Tris/glycine (pH6.8)
0.1%	SDS
3.3% acrylamide: bisacrylamide (30:0.8)	
per 16 ml:	
240 µl	20% ammonium persulfate (APS)
8 µl	TEMED

Resolving gel

380 mM	Tris/glycine (pH 8.8)
0.1%	SDS
10-15%	acrylamide :bisacrylamide (30:0.8)
per 42 ml :	
480 µl	20% APS
12 µl	TEMED

10x SDS-PAGE running buffers (pH 8.3)

250 mM	Tris
1.9 M	Glycine
1%	SDS

3xSDS gel loading buffer

150 mM	Tris/HCl (pH 6.8)
30%	Glycerol
6%	SDS
15%	β-mercaptoethanol
0.3%	bromophenol blue

2.4.2 Coomassie Blue staining of proteins

Following SDS-PAGE, gels were stained in Coomassie Blue solution for at least 30 min with agitation, after which the unbound dye was removed by shaking in a destaining solution.

Coomassie Blue solution

0.1% Coomassie Brillant Blue R250
50% methanol
12% glacial acetic acid
-solution filtered via a Whatman filter

Destaining solution

10% ethanol
7% glacial acetic acid

2.4.3 Drying of SDS-PAGE gels

For permanent recording, SDS-polyacrylamide gels after Coomassie Blue staining were washed in water with agitation with a couple of changes of water to remove the destaining solution. The gels were then shaken in a drying solution for 20 min, after which each gel was placed between 2 dialysis membranes pre-wetted in the drying solution and then air-dried overnight.

Drying solution for polyacrylamide gels

24% ethanol
5% glycerol

2.4.4 Western blotting

Following separation of protein by SDS-PAGE, the proteins were transferred from gels onto nitrocellulose membranes (Schleicher & Schuell BA85) according to the modified protocol of Towbin *et al.* (1979) with the aid of a protein transfer apparatus (Trans-Blot SD, BioRad).

In this “semi-dry” method, the gel and its attached nitrocellulose filter were sandwiched between pieces of Whatman 3MM filter paper which had been soaked in transfer buffer and protein transfer was carried out at RT at 12 V for 40 min, after which the nitrocellulose filter was blocked overnight at 4°C in 5% (W/v) milk powder in 1 x NCP buffer (without sodium azide) and incubated with primary antibody (polyclonal antibody raised against the regulatory domain of DST2 1:500-1:1000 in NCP buffer) for 1 h. After washing several times with NCP buffer, the membrane was incubated with a secondary antibody (diluted 1:10,000 in NCP buffer) for 1h. For the experimental purposes, goat anti-mouse IgG conjugated with peroxidase (Dianova) was used as the secondary antibody. Finally, the membrane was washed a few times with NCP buffer and protein bands were detected via chemiluminescence, by incubating the membrane in enhanced chemiluminescence reagents (ECL, Amersham) for 1 min, and then exposed to X-ray films (X-omat AR5, Kodak).

Transfer buffer

25 mM	Tris/HCl (pH 8.5)
190 mM	Glycine
20%	Methanol
0.02%	SDS

20x NCP buffer (pH 7.2)

48.4 g	Tris
348 g	NaCl
20 ml	Tween 20

-Filled up to 2 l with dH_2O.

Chemiluminescence reagents (light sensitive)

200 µl	Luminol (250 mM in DMSO; Luminol: 3-amino-phthalazinedione)
89 µl	p-Coumaric acid (90 mM in DMSO)
2 ml	Tris/HCl (1M, pH 8.5)

-Filled up to 20 ml with dH_2O.

2.4.5 Bradford assay

Protein quantification was performed according to the method of Bradford (1976). In principle, the Coomassie brilliant blue G250 dye interacts primarily with arginine residues of proteins and causes a shift in the absorbance peak from 465 nm to 595 nm. Bovine serum albumin was used to make a standard colour response curve from which the concentration of protein samples could be determined.

2.4.6 Preparation of actin from rabbit skeletal muscle

Actin was prepared from rabbit skeletal muscle according to the methods described by Spudich and Watt (1971) as well as by Pardee and Spudich (1982). The back and upper thigh muscles of a freshly bled rabbit were sliced into pieces and extracted with high-salt extraction buffer for 10 min with agitation. The mixture was centrifuged (4,000 g, 10 min) and re-extracted to remove myosin, after which the sediment was resuspended in water and the pH adjusted to between 8.2 and 8.5 with 1 M Na_2CO_3 solution. Following centrifugation (4,000 g, 10 min), the supernatant was discarded and the process repeated until swelling of the sediment was observed. The sediment was then washed with cold acetone, dried overnight, extracted once with chloroform and dried again. Finally, the acetone powder was stored at –20ºC for subsequent actin preparation.

10 g of muscle acetone powder were extracted with 200 ml of G-buffer at 0ºC for 30 min, filtered through a nylon net and re-extracted at 0ºC for 10 min. The filtrate was centrifuged (3,000 g, 30 min, 4ºC) and the actin in the supernatant allowed to polymerise for 2 h at 4ºC after addition of KCl (50 mM), $MgCl_2$ (2 mM) and ATP (1 mM). For removal of tropomyosin, solid KCl was then slowly introduced until a final concentration of 0.8 M was reached, and the actin filaments were then sedimented by centrifugation (150,000 g, 3 h, 4ºC). For depolymerisation, the F-actin pellet was dialyzed against several changes of G-buffer and further purified using a Sephacryl S300 gel filtration column (2.5 x 45 cm, Pharmacia). From its optical density at 290 nm, the G-actin concentration could be calculated easily from the ratio of OD_{290}/0.65, whereby 26,000 is the extinction coefficient value of actin at 290 nm (Wegner, 1976). The G-actin prepared could be stored at 4ºC for up to 3 weeks for active applications.

Extraction buffer

0.5 M	KCl
0.1 M	K_2HPO_4

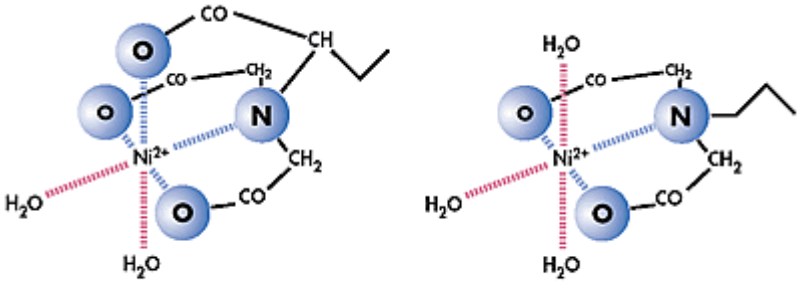
G-buffer (pH 8.0)

2 mM	Tris/ HCl
0.2 mM	$CaCl_2$
0.2 mM	ATP
0.02%	NaN_3
0.5 mM	DTT

2.4.7 Protein purification

2.4.7.1 Purification of Histidine- tagged constructs

Proteins with an affinity tag of six consecutive histidine residues (the 6x His-tag protein) were purified using the Ni-NTA system (nickel-nitrilotriacetic acid, Qiagen). The 6x His tag is much smaller (0.84 kDa) than most other affinity tags and is uncharged at physiological pH. Qiagen Ni-NTA products use the four chelating sites of NTA to bind nickel ions more tightly than alternative metal-chelating purification systems that have only three sites available for interaction with metal ions. The presence of an additional chelation site prevents nickel-ion leaching resulting in greater binding capacity, and also minimizes non-specific binding leading to purer protein preparations.



The catalytic domain of DST2 (amino acid 1-287) was amplified by PCR using DST2-Nt (5'-CGC GGA TCC ATG TCA ACG CTC AAT GTA CC-3') and DST2-Ctcat (5'- GCG CTG CAG ATC TAT TAA TGG TAC CAA GAG TG- 3') and digested with BamHI and PstI, and then inserted into pQE30 vector (Qiagen). The His-tagged fusion protein was expressed in M15 cells. For large prep, 1 L LB-medium was inoculated with a grown 10 ml culture of the construct, grown for another 1-2 h at 37°C and 240 rpm and induced with 0.3 mM IPTG. Expression was carried out for 2 h at 37°C and 240 rpm. Cells were harvested (10 min, 5000 rpm, sorvall rotor GSA, 4°C). Expressed proteins were purified according to the Qiagen protocol.

2.4.7.2 Purification of maltose-binding-protein (MBP) tagged constructs

In the protein fusion and purification system, the cloned gene is inserted into a pMAL vector (NEB) down stream from the malE-gene, which encodes maltose-binding protein (MBP). This results in the expression of an MBP-fusion protein. The technique uses the strong tac-promoter and the translation initiation signals of MBP to express large amounts of the fusion protein. The fusion protein is then purified by one-step affinity purification for MBP. pMAL-c2 vector has an exact deletion of the malE signal sequence, resulting in cytoplasmic expression of the fusion protein. Between malE sequence and the polylinker there is a spacer sequence coding for 10 asparagine residues. This spacer insulates MBP from the protein of interest, increasing the chances that a particular fusion will bind tightly to the amylose resin.
The N-terminal catalytic domain (amino acid 1-287), the C-terminal regulatory domain (amino acid 288-461) and the full-length cDNA of DST2 were amplified using primers DST2-Nt and DST2-Ctcat, DST2-Ntreg and DST2-Ct, DST2-Nt and DST2-Ct, respectively, and cut with BamHI and Pst1, and were inserted into pMAL-c2 vector (NEB). Maltose binding protein-tagged fusion proteins were expressed in M15 [pREP4] cells. The recombinant proteins were purified with the protocol described in the NEB catalogue. 1L LB (including 0.2% glucose) was inoculated with a grown 10 ml culture of cells containing the fusion plasmid. Glucose is necessary in the growth medium to repress the maltose genes on the chromosome of the *E. coli* host. Cells were grown to A_{600} ~ 0.7 at 37°C and induced with 0.3 mM IPTG. Expression was carried out at 37°C for 2 hours. Cells were harvested by centrifugation at 4000xg for 20 min and the supernatant was discarded.

The pellet was re-suspended in 50 ml column buffer. Samples were frozen at -20ºC overnight and thawed in ice-water. Cells were sonicated and debris was spun down (9000 x g for 30 min). Amylose resin column was washed with 8 column volumes of column buffer. The supernatant of the centrifugation was loaded on the amylose resin column and the column was washed with 12 volumes of column buffer. Fusion proteins were eluted with column buffer + 10 mM maltose.

Column buffer

20 mM	Tris/HCl (pH 7.4)
200 mM	NaCl
1 mM	EDTA
1 mM	Azide
1 mM	DTT (or β-mercaptoethanol)

2.4.8 Low shear viscometry

Low shear viscometry was performed after 20 min of incubation at 25ºC in a falling ball viscometer (MacLean-Fletcher and Pollard, 1980). The reaction mixture (160 µl) usually contained 0.5 mg/ml rabbit skeletal muscle actin, and polymerisation was inhibited by the addition of G-actin to buffered $MgCl_2$ (final concentration: 2 mM $MgCl_2$, 10 mM imidazole, pH 7.2, 1 mM ATP, 0.2 mM $CaCl_2$, or 1 mM EGTA). The data shown are the average values of duplicate experiments.

2.4.9 Severing activity of severin measured by fluorescence spectroscopy

Pyrene-labelled actin (8 µM) was polymerised for 15 min in G-buffer (2 mM Tris/HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.01% NaN_3, 0.5 mM DTT, 2 mM $MgCl_2$) for use in the fluorescence measurements carried out with an Aminco Bowman luminescence spectrometer (Sopra GmbH, Buettelborn, FRG). All measurements were done in a sample volume of 800 µl at 25ºC with an excitation wavelength of 365 nm and an emission wavelength of 386 nm in G-buffer and at a final actin concentration of 0.8 µM. The slow depolymerisation of actin filaments is dramatically increased if severing activity raises the number of pointed ends.

Individual experiments were performed in duplicates. Measured relative fluorescence values were plotted against time and the slopes of these plots were calculated in the linear range of fluorescence decrease which were subsequently plotted versus increasing concentration of the severin.

2.4.10 Inhibition of actin depolymerisation analysed by fluorescence spectroscopy

Inhibition of actin depolymerisation was examined with the aid of pyrene-labelled G-actin (8 μM) using a luminescence spectrometer (Sopra GmbH) in a final reaction volume of 800 μl at 25ºC. Highly active construct of DST2 (1-326) was incubated with severin or without severin in the kinase buffer (20 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol, 10 mM β-glycerolphosphate, 10 mM $MgCl_2$ containing 0.1 mM ATP) for 20 min and pyrene-labelled G-actin was added. All measurements were performed at an excitation wavelength of 365 nm and an emission wavelength of 386 nm, and the fluorescence units were subsequently plotted against time. Data were confirmed with duplicate experiments. Measured relative fluorescence values were plotted against time and the slopes of these plots were calculated in the linear range of fluorescence decrease, which were subsequently plotted versus increasing concentration of severin.

2.4.11 *In vitro* kinase assay

Kinase reaction with purified recombinant DST2 was performed in kinase buffer (20 mM Tris/HCl, pH 7.5, 1 mM DTT, 10 mM β-glycerol triphosphate, 10 mM $MgCl_2$) and 0.1mM ATP (2-5μCi of ^{32} P-ATP). The reaction was incubated for 10 - 30 min for 30ºC. Reactions were terminated by the addition of 3 x SDS sample buffer and boiling for 5 min. A portion of the sample (15μ) was separated on a 12% SDS-polyacrylamide gel. The gel was then fixed and autoradiographed.

Chapter 3

RESULTS

3.1 Molecular Characterisation of DST2

3.1.1 Sequence analysis

DST2 was discovered by screening the sequences of the *D. discoideum* cDNA project database in Tsukuba, Japan (website: http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html) using DST1 as query. The genomic sequence of DST2 was amplified by PCR, cloned into the pQE30-vector and sequenced. Analysis of the genomic structure of the DST2 gene showed that it is composed of five exons separated by four introns and spans a genomic region of approximately 1.8kb. Introns are usually very small and AT-rich in *D. discoideum*. These features are also apparent in the four DST2 introns which have sizes of 77, 119, 77 and 175bp, respectively. The sequences of all exon/intron borders follow the consensus 'GT-AG' rule (Fig. 3).

The full length cDNA clone of DST2 was kindly provided by the *D. discoideum* cDNA project in Tsukuba. The cDNA contains an open reading frame of 1383bp that encodes a protein of 461 amino acids with a predicted molecular mass of 52 kDa. Motif searches and sequence comparison showed that DST2 consists of a 287-amino acid N-terminal kinase domain and a 174-amino acid C-terminal regulatory domain. Database searches revealed that DST2 is a member of the STE20 (sterile 20) or PAK (p21 activated kinase) family of protein kinases. DST2 displays high homology throughout the kinase domain and contains the amino acid sequence GTPYFWMAPEV in the kinase domain (Fig. 4). This sequence motif, the so-called PAK signature is characteristic for the PAK / STE20 family of protein kinases and is critical for kinase activity of STE20 (Wu *et al*., 1995).

1 ACGCGTCCGA AATAAATCAA AAAGAAA*ATG* TCAACGCTCA ATGTACCAAA AGAGACAATG
61 AGTAGAAAAG ACCCAGAAAA GTTTTTCACT ATTGTTGAGA AATTGGGTGA AGG**GT**AAGTG
121 AAATCTATTT TAAATTTTTT TTTTTTTTTT TTTTTTTTGC TCTGTTCTAA CACAATAATA
181 ATAAATAT**AG** TTCATATGGT TCAGTATATA AAGCAATTAA TATTTCAACA GGAATTGTTG
241 TTGCCATTAA AAAGGTATCA GTCGACAATG ATCTTGAAGA TATGGAAAAG GAAATCAGCT
301 TTATGAAACA ATGTAAGAGT CCATACATTG TAACTTACTA TGCAAGCTTT AGAAAAGAAA
361 ATGAAGTTTG G**GT**ACATATA TATATATATA AATATTTCTA ACATTAATAT ACATTTATTT
421 ATTTTATATA TATTAATATT TTAAAAATTT ATTTCTTTTT TTTAAAAAAA AAAAAAAAAA
481 AAAAAATT**AG** ATTGTTATGG AACATTGTGG AGCAGGATCA GTATGTGATG CAATGAAAAT
541 TACAGATAAG ACATTATCAG AGGATCAAAT TGCAGTTGTT AGTAGGGATG TTTTACAAGG
601 TTTAGCATAT TTACATTCAG TTAGAAAGAT TCATAGAGAT ATTAAAGCAG GTAACATTTT
661 AATGAATCAT AAAGGTGAAT CGAAATTAGC AGATTTCGGT GTTAGTGGTC AATTATCAGA
721 TACAATGGCA AAACGTCAAA CTGTAATCGG TACCCCATTT TGGATGGCTC CTGAAGTCAT
781 TCAAGAAATT GGTTATGATT ATAAA**GT**ATG TAATTTATTT ATAGATAATA TATATATATA
841 TATATTTTAT TTTTTATTAA TTTGATATTA TTATTATTAT **AG**GCAGATAT TTGGTCATAT
901 GGTATTACAT GTATTGAAAT GGCAGAATCA AAACCACCAT TATTTAATGT TCATCCAATG
961 AGAGTCATAT TTATGATTCC AAATCCATCA AGACCACCAC CAAAATTAAC AGAACCAGAG
1021 AAATGGTCAC CAGAATTCAA TGACTTTTTA GCAAAATGTT TAACAAGAAA ACCAGAATTA
1081 AGACCTTCCG CTGAGGAATT ATTAAAACAT CCATTCATTA CAAAAGCAAA ATCACATTCA
1141 CTCTTGGTAC CATTAATAGA TGAACAAGAT ATAATCATCA ATGAAAAAGG TAGAGAAGTC
1201 GCTTTAGGTA TTGAACAAAG AGATGAAGAA GAGGAAGATG AAGATGAAGA TTCTGAAGAT
1261 TCTGATGATA ATAGA**GT**AAT ATTATCATTA TCATTATTAT TATTATTATT ATTATTATTA
1321 TTATTATTAT TATTATTATT ATTATTATTA TTATTATTAT TATTATTATT ATTATTATTA
1382 TTATTATTAT TATTATTATT ATTTATATAA TAAACTAATT TATTTATTTT TTATTTTTAT
1441 TTTATTTT**AG** GGAACTATGG TTAGAGCGAA ACCAAGATCA ATGCAAAATT CAGGTGGTGA
1501 AGATAATGAT GAAGAATATG ATACAGGTAC AATGGTTATT ACTGATAATA AGAATTCTTA
1561 TGATACAGTT GTATTTAATA ATGATGATGA AGATAGTGGA ACAATGAAAT TAAAGAATAC
1621 AATGCCTTCA AATAAAAAGA ATTTTGTACC AGATTATATG AATCAATTTA AAAAGAGTGA
1681 TGATGATGTC ACCAATGTTC CTTTAAGTGA TAAATACTCT AGTTATTCCT TAGAGGAATT
1741 AAAGAAAATG TTGGCTGAAT TAGAAATTGA AAGAGAAAAA GAAGTTCAAA AAACACTTGA
1801 AAAATTTTCA ATTAATCGTC AAGCTTTATT AGCTGTAATT GATGAAAAGA AATCAAAG*TA*
1861 *GTAG*TA

Fig. 3 Genomic sequence of DST2.
The gene consists of five exons and four short introns (77, 119, 77 and 175bp, respectively). Intron sequences are underlined and consensus sequences for 5' (GT) and the 3' splice site (AG) are shown in **bold**. The start and stop codons are indicated in *italic*. Numbering of nucleotides is indicated on the left side.

```
1      MSTLNVPKET  MSRKDPEKFF  TIVEKLGEGS  YGSVYKAINI  STGIVVAIKK  VSVDNDLEDM
61     EKEISFMKQC  KSPYIVTYYA  SFRKENEVWI  VMEHCGAGSV  CDAMKITDKT  LSEDQIAVVS
121    RDVLQGLAYL  HSVRKIHRDI  KAGNILMNHK  GESKLADFGV  SGQLSDTMAK  RQTVIGTPFW
181    MAPEVIQEIG  YDYKADIWSY  GITCIEMAES  KPPLFNVHPM  RVIFMIPNPS  RPPPKLTEPE
241    KWSPEFNDFL  AKCLTRKPEL  RPSAEELLKH  PFITKAKSHS  LLVPLIDEQD  IIINEKGREV
301    ALGIEQRDEE  EEDEDEDSED  SDDNRGTMVR  AKPRSMQNSG  GEDNDEEYDT  GTMVITDNKN
361    SYDTVVFNND  DEDSGTMKLK  NTMPSNKKNF  VPDYMNQFKK  SDDDVTNVPL  SDKYSSYSLE
421    ELKKMLAELE  IEREKEVQKT  LEKFSINRQA  LLAVIDEKKS  K
```

Fig. 4 Deduced amino acid sequence of DST2 in standard single-letter code.
The sequence is composed of two domains, an N-terminal kinase domain (aa 1-287) and a C-terminal domain of predicted regulatory function (aa 288-461). The kinase domain is underlined. The STE20- or PAK signature is shaded. Numbering of amino acids is indicated on the left side.

3.1.2 Sequence comparison with STE20-like protein kinases

The STE20-like protein kinase family can be divided into two subfamilies based on their domain structure and regulation. The PAK / STE20 subfamily contains a C-terminal catalytic domain and an N-terminal binding site for the small G proteins Cdc42 and Rac1, which are considered to be key regulatory molecules linking surface receptors to the organization of the actin cytoskeleton (Herskowitz *et al*., 1995; Hall *et al*., 1998). In contrast, the kinases of the second subfamily, the GCK subfamily, have an N-terminal kinase domain and a C-terminal regulatory domain that does not have a recognizable GTPase binding site. However, both subfamilies are highly conserved throughout the catalytic domain.

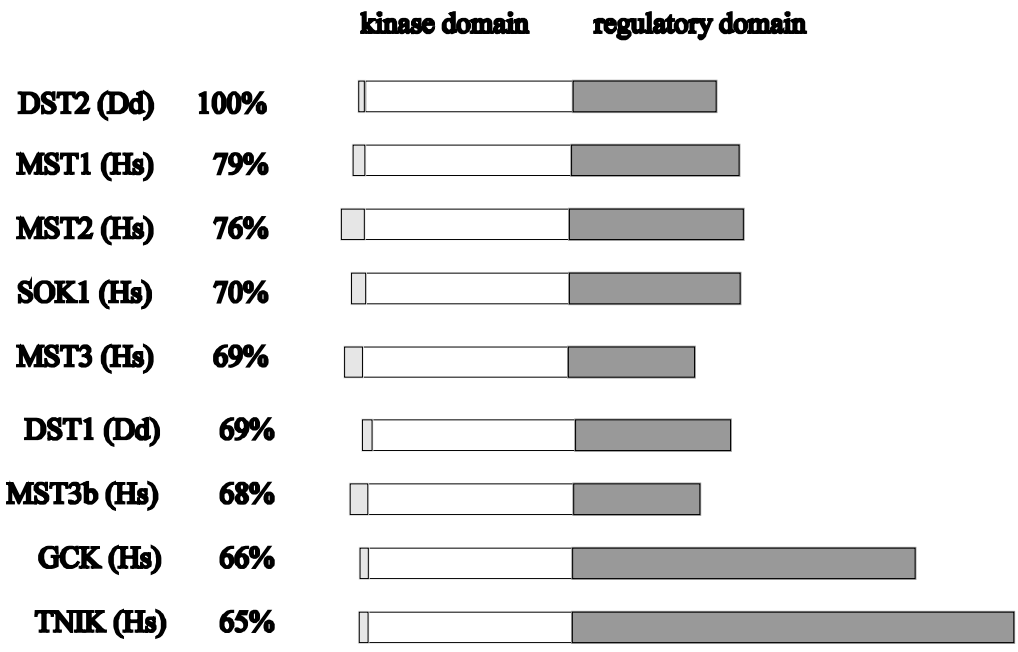
Sequence analysis showed that the overall structure of DST2 more closely resembles the GCK subfamily of the STE20-like kinase family. We aligned the amino acid sequence of the DST2 kinase domain with other members of the GCK subfamily (Fig. 5).

Fig. 5. Sequence alignment of DST2 with other members of the GCK subfamily.
The alignment of the predicted amino acid sequence of the DST2 kinase domain with the corresponding domains of MST1, MST2, SOK1, MST3, DST1, MST3b, GCK, TNIK is shown. The sequence alignment was done with the program Clustal from the UWGCG program package. The conserved Lysine (K) of the catalytic domains, which is crucial for ATP binding, is indicated in red. The PAK / STE20 signature sequence is indicated in blue. Amino acids which are conserved in six or more of the proteins are highlighted in yellow. The abbreviations used are: Dd, *D. discoideum*; Hs, *Homo sapiens*.

```
DST2 (Dd)   DPEKFFTIVE KLGEGSYGSV YKAINISTGI VVAIKKVSV. ..DNDLEDME
MST1 (Hs)   QPEEVFDVLE KLGEGSYGSV YKAIHKETGQ IVAIKQVPV. ..ESDLQEII
MST2 (Hs)   QPEEVFDVLE KLGEGSYGSV FKAIHKESGQ VVAIKQVPV. ..ESDLQEII
SOK1 (Hs)   DPEELFTKLD RIGKGSFGEV YKGIDNHTKE VVAIKIIDLE EAEDEIEDIQ
MST3 (Hs)   DPEELFTKLE KIGKGSFGEV FKGIDNRTQK VVAIKIIDLE EAEDEIEDIQ
DST1 (Dd)   DPEELYVRQE KIGKGSFGEV FKGINKKTNE TIAIKTIDLE DAEDEIEDIQ
MST3B(Hs)   DPEELFTKLE KIGKGSFGEV FKGIDNRTQK VVAIKIIDLE EAEDEIEDIQ
GCK  (Hs)   DPRDRFELLQ RVGAGTYGDV YKARDTVTSE LAAVKIVKLD PG.DDISSLQ
TNIK (Hs)   DPAGIFELVE LVGNGTYGQV YKGRHVKTGQ LAAIKVMDVT GDEE..EEIK

DST2 (Dd)   KEISFMKQ.C KSPYIVTYYA SFRK...... ENEVWIVMEH CGAGSVCDAM
MST1 (Hs)   KEISIMQQ.C DSPHVVKYYG SYFK...... NTDLWIVMEY CGAGSVSDII
MST2 (Hs)   KEISIMQQ.C DSPYVVKYYG SYFK...... NTDLWIVMEY CGAGSVSDII
SOK1 (Hs)   QEITVLSQ.C DSPYITRYFG SYLK...... STKLWIIMEY LGGGSALDLL
MST3 (Hs)   QEITVLSQ.C DSPYVTKYYG SYLK...... DTKLWIIMEY LGGGSALDLL
DST1 (Dd)   QEINVLSQ.C ESPFVTKYFG SFLK...... GSKLWIIMEY LAGGSVLDLM
MST3B(Hs)   QEITVLSQ.C DSPYVTKYYG SYLK...... DTKLWIIMEY LGGGSALDLL
GCK  (Hs)   QEITILRE.C RHPNVVAYIG SYLR...... NDRLWICMEF CGGGSLQEIY
TNIK (Hs)   QEINMLKKYS HHRNIATYYG AFIKKNPPGM DDQLWLVMEF CGAGSVTDLI

DST2 (Dd)   KITDK.TLSE DQIAVVSRDV LQGLAYLHSV RKIHRDIKAG NILMNHKGES
MST1 (Hs)   RLRNK.TLTE DEIATILQST LKGLEYLHFM RKIHRDIKAG NILLNTEGHA
MST2 (Hs)   RLRNK.TLIE DEIATILKST LKGLEYLHFM RKIHRDIKAG NILLNTEGHA
SOK1 (Hs)   K  PG PLEE TYIATILREI LKGLDYLHSE RKIHRDIKAA NVLLSEQGDV
MST3 (Hs)   E..PG.PLDE TQIATILREI LKGLDYLHSE KKIHRDIKAA NVLLSEHGEV
DST1 (Dd)   K..PG.PFDE GYIAIILREL LKGLEYLHSE GKIHRDIKAA NVLLSASGDV
MST3B(Hs)   E..PG.PLDE TQIATILREI LKGLDYLHSE KKIHRDIKAA NVLLSEHGEV
GCK  (Hs)   HAT..GPLEE RQIAYVCREC RQGLHHLHSQ GKIHRDIKGA NLLLTLQGDV
TNIK (Hs)   KNTKGNTLKE EWIAYICREI LRGLSHLHQH KVIHRDIKGQ NVLLTENAEV

DST2 (Dd)   KLADFGVSGQ LSDTMAKRQT VIGTPFWMAP EVI.....QE IGYDYKADIW
MST1 (Hs)   KLADFGVAGQ LTDTMAKRNT VIGTPFWMAP EVI.....QE IGYNCVADIW
MST2 (Hs)   KLADFGVAGQ LTDTMAKRNT VIGTPFWMAP EVI.....QE IGYNCVADIW
SOK1 (Hs)   KLADFGVAGQ LTDTQIKRNT FVGTPYWMAP EVI     KQ SAYDFKADIW
MST3 (Hs)   KLADFGVAGQ LTDTQIKRNT FVGTPFWMAP EVI.....KQ SAYDSKADIW
DST1 (Dd)   KLADFGVSGQ LTDQMTKRNT FVGTPFWMAP EVI.....KQ TGYDSKADIW
MST3B(Hs)   KLADFGVAGQ LTDTQIKRNT FVGTPFWMAP EVI.....KQ SAYDSKADIW
GCK  (Hs)   KLADFGVSGE LTASVAKRRS FIGTPYWMAP EVAAVE..RK GGYNELCDVW
TNIK (Hs)   KLVDFGVSAQ LDRTVGRRNT FIGTPYWMAP EVIACDENPD ATYDFKSDLW

DST2 (Dd)   SYGITCIEMA ESKPPLFNVH PMRVIFMIPN PSRPPPKLTE PEKWSPEFND
MST1 (Hs)   SLGITAIEMA EGKPPYADIH PMRAIFMI.. PTNPPPTFRK PELWSDNFTD
MST2 (Hs)   SLGITSIEMA EGKPPYADIH PMRAIFMI.. PTNPPPTFRK PELWSDDFTD
SOK1 (Hs)   SLGITAIELA KGEPPNSDLH PMRVLFLI    PKNSPPTLE   GQHSKPFKE
MST3 (Hs)   SLGITAIELA RGEPPHSELH PMKVLFLI.. PKNNPPTLE. .GNYSKPLKE
DST1 (Dd)   SMGITALEMA KGEPPRADLH PMRALFLI.. PKDPPPTLE. .GNFSKGFKE
MST3B(Hs)   SLGITAIELA RGEPPHSELH PMKVLFLI.. PKNNPPTLE. .GNYSKPLKE
GCK  (Hs)   ALGITAIELG ELQPPLFHLH PMRALMLMSK SSFQPPKLRD KTRWTQNFHH
TNIK (Hs)   SLGITAIEMA EGAPPLCDMH PMRALFLI.. PRNPAPRL.K SKKWSKKFQS

DST2 (Dd)   FLAKCLTRKP ELRPSAEELL KHPFI.TKAK SHSLLVPLID
MST1 (Hs)   FVKQCLVKSP EQRATATQLL QHPFV.RSAK GVSILRDLIN
MST2 (Hs)   FVKKCLVKNP EQRATATQLL QHPFI.KNAK PVSILRDLIT
SOK1 (Hs)   FVEACLNKDP RFRPTAKELL KHKFITRYTK KTSFLTELID
MST3 (Hs)   FVEACLNKEP SFRPTAKELL KHKFILRNAK KTSYLTELID
DST1 (Dd)   FCALCLNKDP NQRPTAKDLL KHKFI.KAAK KTSSLTDLIE
MST3B(Hs)   FVEACLNKEP SFRPTAKELL KHKFILRNAK KTSYLTELID
GCK  (Hs)   FLKLALTKNP KKRPTAEKLL QHPFTTQQL. PRALLTQLLD
TNIK (Hs)   FIESCLVKNH SQRPATEQLM KHPFIRDQPN ERQVRIQLKD
```

The alignment clearly showed that the DST2 kinase domain is highly homologous to the kinase domains of MST1 (Hs), MST2 (Hs), SOK1 (Hs), MST3 (Hs), DST1 (Dd), MST3b (Hs), GCK (HS) and TNIK (Hs). These proteins share a similar structure and have 79, 76, 70, 69, 69, 68, 66 and 65% amino acid similarities in the catalytic domains, respectively. These proteins have a highly conserved catalytic domain in common but differ in their C-terminal regulatory domain, which does not contain any identifiable sequence motifs. Interestingly, DST2 is more closely related to human MST1 and MST2 (79% and 76% similarity I in the catalytic domain, respectively) than to *D. discoideum* DST1 (with only 69%) (Fig. 6A). To further clarify the relationships between these protein kinases and DST2, we calculated multiple sequence alignments of the catalytic domains. The derived evolutionary tree is split into two main branches, one formed by MST1, MST2, DST2, GCK and TNIK, the other one by MST3, MST3b, DST1 and SOK-1 (Fig. 6B)

A



B

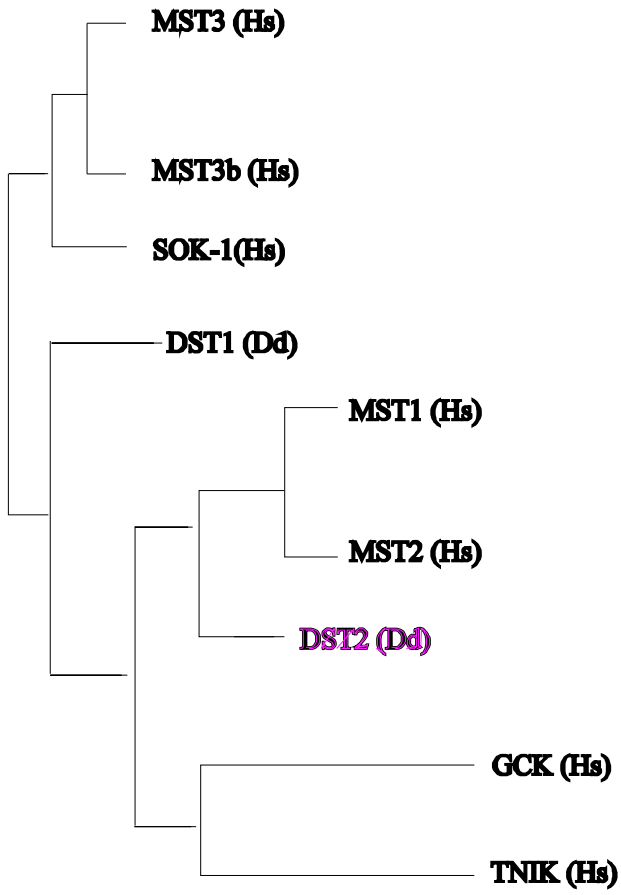
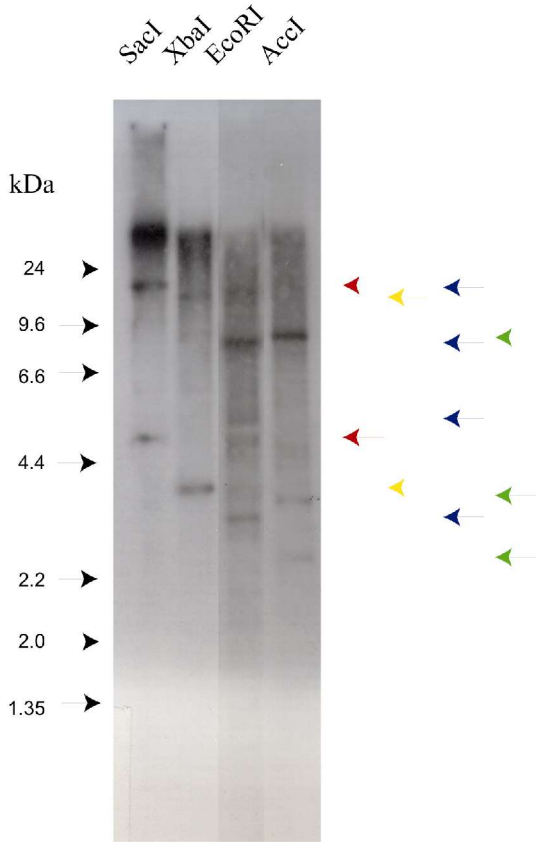


Fig. 6. Topology of DST2 (A) and evolutionary tree of the GCK subfamily (B).
A: The structures of DST2 and eight GCK subfamily members are schematically depicted. The sequence similarities of the catalytic domains were calculated relative to the catalytic domain of DST2. B: Evolutionary tree of GCK subfamily members. A multiple sequence alignment of the catalytic domains of GCK subfamily members was calculated with the program Clustal of the UWGCG package (University of Wisconsin Genetic Computer Group). The alignment was used to construct a phylogenetic tree with the programs Protdist and Kitch of PHYLIP (Phylogeny Inference Package), version 3.5c, by Joseph Felsenstein from the University of Washington, Dd, *D. discoideum*; Hs, *Homo sapiens*.

3.1.3 Southern analysis of DST2

To investigate whether the DST2 gene exists as a single copy in the *Dictyostelium* genome, genomic DNA of AX2 cells was digested with different restriction enzymes and the digested DNA fragments were hybridized with radioactively labeled full length genomic DNA of DST2. Under stringent conditions we detected two bands in the SacI digest, two in the XbaI digest, four bands in the EcoRI digest and three bands in the AccI digest (Fig. 7). There are no SacI and XbaI sites in the genomic DNA of DST2, yet 2 fragments were found after digestion with SacI or XbaI. The strong band in the SacI digest and the weaker one in the XbaI digest in the range of 50kb represent sheared undigested genomic DNA and are therefore not taken into account. Furthermore, after cutting with EcoRI four large fragments (app. 15, 8, 5 and 3kb) were detectable, even though we expected only two large and one small fragment (ca. 0.5kb). The small fragment of 0.5kb most likely escaped detection. Three bands were detected with AccI, although only one AccI site exists. The same band pattern was obtained when using a 260bp fragment derived from the regulatory region of DST2 for hybridisation (data not shown). Taken together, these data indicate the presence of two copies of DST2 in the *Dictyostelium* genome.

A



B

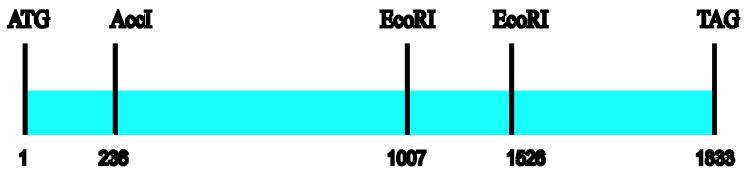
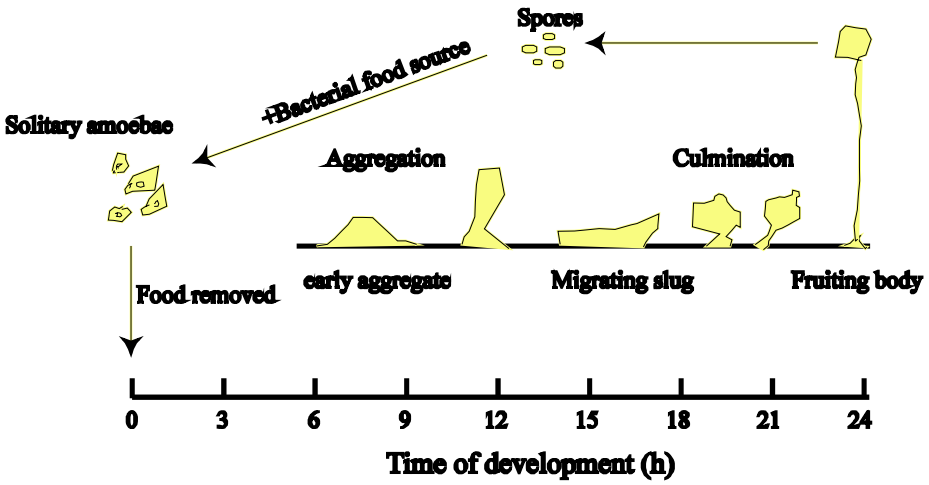


Fig. 7 Southern blot analysis of DST2.
A: Genomic DNA of AX2 cells was digested with SacI, XbaI, EcoRI, or AccI. The fragments were separated in a 0.8% agarose gel and blotted onto nitrocellulose. DST2 specific fragments were detected with the full length ^{32}P-labeled 1.8 kb DST2 genomic DNA. The detected bands are marked with differently coloured arrows (red: SacI; yellow: XbaI; blue: EcoRI; green: AccI). Sizes of the DNA marker are shown on the left side. B: Schematic representation of AccI and EcoRI restriction sites in the genomic DNA of DST2. The positions of the start, stop codon and of the restriction sites are shown.

3.1.4. Northern analysis of DST2

There are two distinct phases in the life cycle of *D. discoideum,* growth and development (Bonner *et al*., 1967; Loomis *et al*., 1975). Amoebae continue to grow as long as a bacterial substrate is available. Development is initiated when the bacterial food supply is exhausted. Then amoebae aggregate by chemotaxis and pass through various morphogenetic stages (Fig. 8A). Developmental regulation has been demonstrated for a number of *D. discoideum* genes.
To determine whether DST2 transcription is regulated during development, we have performed Northern Blot analysis. Total RNA from different stages of development of *D. discoideum* strain AX2 was isolated, separated, blotted onto nitrocellulose and hybridized with radioactively labeled full length cDNA of DST2. Under high stringency conditions, a single 1.5kb mRNA of DST2 was detected. We found that the DST2 transcript is expressed throughout development, but strongly upregulated at 12h of development. To confirm these results, the blot was reprobed using severin and Contact site A probes (Fig. 8B). As expected, severin was expressed at t_0 and throughout development, while mRNA level of Contact site A, a plasma membrane glycoprotein required for cell adhesion during aggregation, was found highest at 9h and began to fall at 12h of development, confirming published results (André *et al*., 1988; Siu *et al*., 1988). These results clearly show that DST2 transcript is accumulated at 12 h of the development of *Dictyostelium,* at the time when amoebae enter the slug stage.

A



B

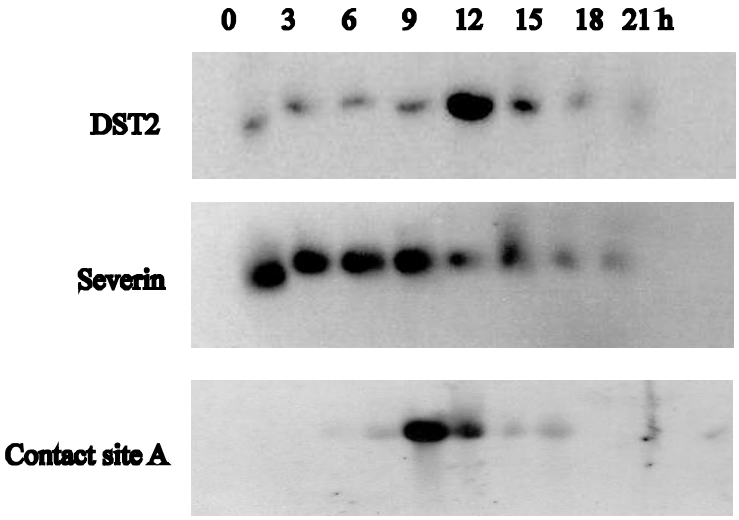
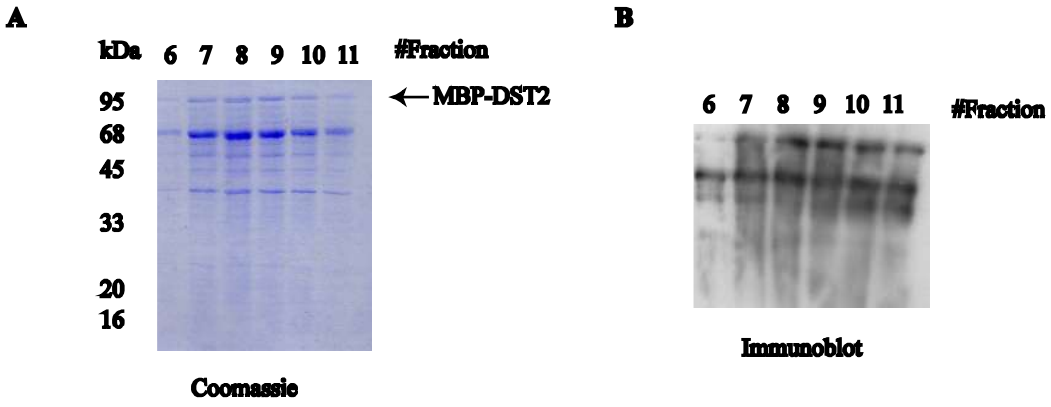


Fig. 8 Northern analysis of DST2
A: Morphological stages in the development of *D. discoideum*. The time course of development is for standard conditions as described by Sussman (1996). B: Total RNA from different stages of development (indicated in hours) was probed with a radioactively labeled cDNA fragment coding full length of DST2. Severin and Contact site A were used as positive controls.

3.2 Biochemical and cell biological characterization of DST2

3.2.1 Expression and purification of recombinant DST2

Full length DST2 (MBP-DST2), the catalytic domain (MBP-DST2-cat) and the regulatory domain (MBP-DST2-reg) were expressed as maltose binding protein (MBP)-tagged fusion proteins. After opening the bacteria, the recombinant proteins were found soluble and purified using affinity chromatography. Recombinant MBP-DST2 showed a molecular mass of about 95 kDa, MBP-DST2-cat of about 80 kDa and MBP-DST2-reg of about 70 kDa (Fig. 9A, C, E). Expression and purification worked very well for MBP-DST-reg, while considerable degradation was observed in the case of MBP-DST2 and, less pronounced, MBP-DST2-cat. The purified recombinant proteins were used for the generation of polyclonal antisera as well as for the investigation of their catalytic activity in *in vitro* assays (see 3.2.3). The full length proteins as well as the degradation products were also seen in immunoblots using the obtained polyclonal antisera (Fig. 9 B, D). The polyclonal antiserum against MBP-DST2-reg was superior and used for further investigations. However, the affinity and specificity of the anti MBP-DST2-reg antibodies was not sufficient for immunoprecipitation of native DST2 from whole *Dictyostelium* cell homogenates.



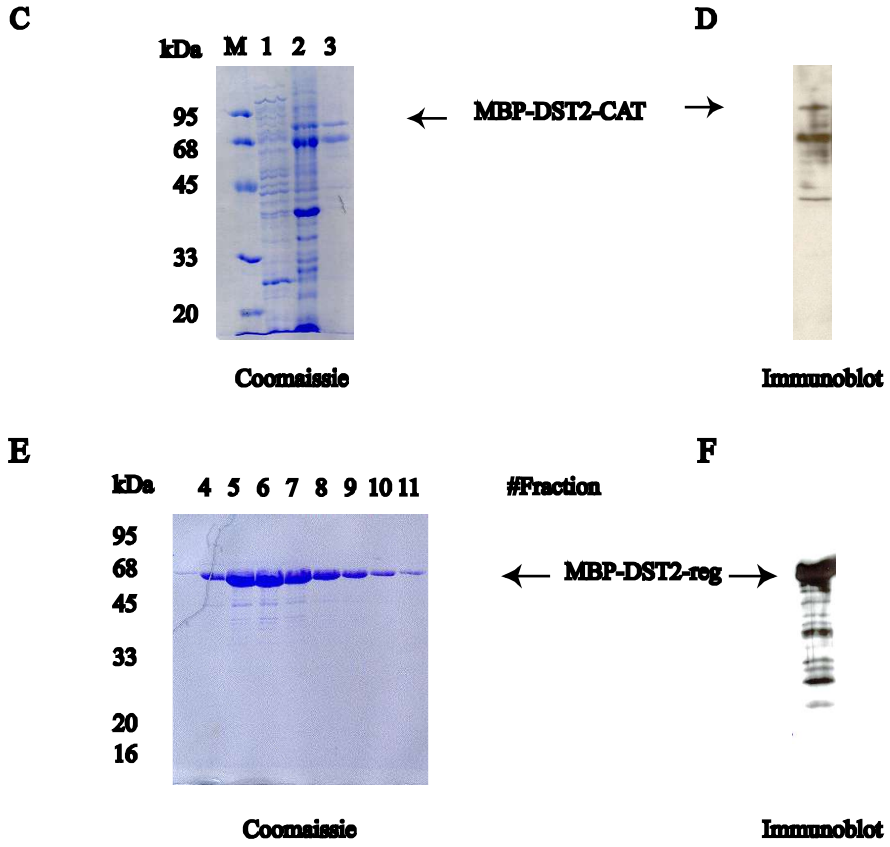


Fig. 9 Purification of recombinant full length, regulatory and catalytic domains of DST2.
A: Full length recombinant DST2 was expressed as MBP-tagged fusion protein (ca. 95 kDa). B: Immunoblotting analysis of recombinant DST2 using a polyclonal antiserum against the regulatory domain. C: The recombinant catalytic domain of DST2 showed a molecular mass of about 80 kDa. Crude extract (1), insoluble matter (2), purified recombinant catalytic domain (3). D: Immunobldtting analysis of recombinant catalytic domain using a polyclonal antiserum against the catalytic domain. E: The regulatory domain was expressed as MBP-tagged fusion protein (ca. 70 kDa). F: The recombinant regulatory domain was detected using a polyclonal antiserum against the regulatory domain.

3.2.2 Western analysis of DST2

The expression of DST2 in AX2 wild type cells was examined using western blot analysis. A polyclonal antiserum (SA7652), raised against the regulatory domain of DST2, recognized a protein of about 63 kDa during all stages of development. The amount of protein was lower at t_0 and t_6 and increased until t_{21} (Fig. 10). The deduced amino acid sequence predicted a molecular mass of 52 kDa for DST2. Compared to that, we observed a much higher apparent molecular mass of 63 kDa. This could be due to either post-translational modifications or reduced mobility of the polypeptide in SDS-PAGE. Similar differences in apparent and calculated molecular mass were reported for the related kinases MST1, MST2 and DST1. MST1 and 2 have an estimated molecular mass of 61 and 63 kDa in SDS-PAGE while the predicted molecular mass is 55.6 kDa for MST1 and 56.3 kDa for MST2 (Taylor *et al*., 1996). DST1 also showed an apparent molecular mass of 62 kDa in SDS-PAGE, whereas the calculated molecular mass is about 53 kDa (Eichinger *et al*., 1998).

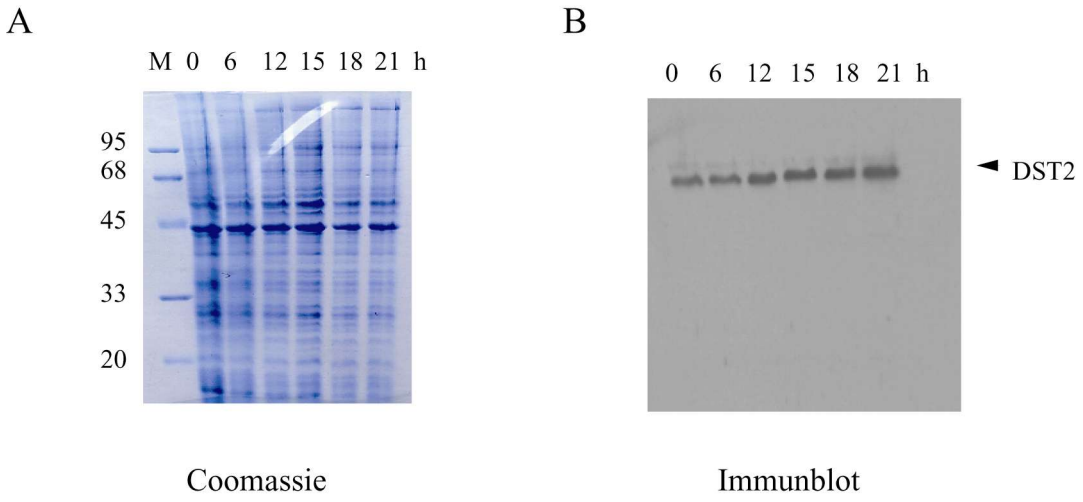


Fig. 10 Expression of DST2 in AX wild type cells and Western blot analysis.
A: Wild-type *Dictyostelium* AX2 cells were cultivated on SM plates at 21°C for 21 hours. Cells were harvested from different developmental stages and total cell homogenates were separated on 12% SDS-PAGE and stained with Coomassie Blue. B: Western blot analysis using an antiserum against the regulatory domain of DST2 as primary antibody. Time of development in hours is indicated above the gel and blot, respectively.

3.2.3 *In vitro* kinase assays with recombinant DST2 constructs

Bacterially expressed fusion proteins of MBP and full length DST2 (MBP-DST2), the catalytic domain (MBP-DST2-cat) and the regulatory domain (MBP-DST2-reg) were used in *in vitro* kinase assays either alone or with myelin basic protein (MBP') as substrate. As shown in Fig. 11, MBP-DST2 was able to phosphorylate itself (lane 1) and MBP' (lane 2). However, MBP-DST2-cat (lanes 3 and 4) and MBP-DST2-reg (lanes 5 and 6) did not show any kinase activity. The loss of catalytic activity of the recombinant catalytic domain was unexpected and could be due to a protein-folding problem.

Since full length recombinant DST2 protein was tagged with maltose binding protein, we examined whether maltose binding protein might be phosphorylated by DST2 or have any effect on kinase activity. As shown in Fig. 12, MBP was not phosphorylated by MBP-DST2 and no difference in the autophosphorylation of DST2 was seen in the presence of MBP (lane 1, lane 2), while MBP-DST2 still phosphorylated MBP' (lane 3). These results show that added MBP is not phosphorylated by MBP-DST2 and does not influence the catalytic activity of MBP-DST2. MBP-DST2 phosphorylated itself, but it was not clear where the autophosphorylation occurs. To find out which domain of DST2 is phosphorylated during autophosphorylation, the recombinant regulatory and catalytic domains of DST2 were used as potential substrates in the *in vitro* kinase assay. While the regulatory domain was heavily phosphorylated by MBP-DST2 (lane 4), we observed no phosphorylation of the catalytic domain (lane 5), indicating that the phosphorylation site(s) of autophosphorylation are located on the regulatory domain.

In addition to MBP', we tested several additional potential substrates of MBP-DST2 in *in vitro* kinase assays. H1 and α-casein turned out to be poor substrates for MBP-DST2 (data not shown). We also tested *Dictyostelium* severin as a potential substrate for MBP-DST2, because previously it was shown that severin was very efficiently phosphorylated by DST1 (Eichinger *et al*., 1998) and found that severin is also a very good substrate for MBP-DST2 (Fig. 13). All further kinase assays were carried out with either no substrate or using MBP' or severin as a substrate.

To address the question whether autophosphorylation on the regulatory domain could regulate the kinase activity, we incubated MBP-DST2 in the presence of ^{32}P-ATP for 0 or 20 min followed by the addition of severin as a substrate.

Pre-incubation of DST2 with ^{32}P-ATP led to a strong increase in the autophoshorylation level, however, the phosphorylation level on severin was not changed. These results suggest that autophosphorylation of DST2 does not increase its kinase activity (Fig. 13).

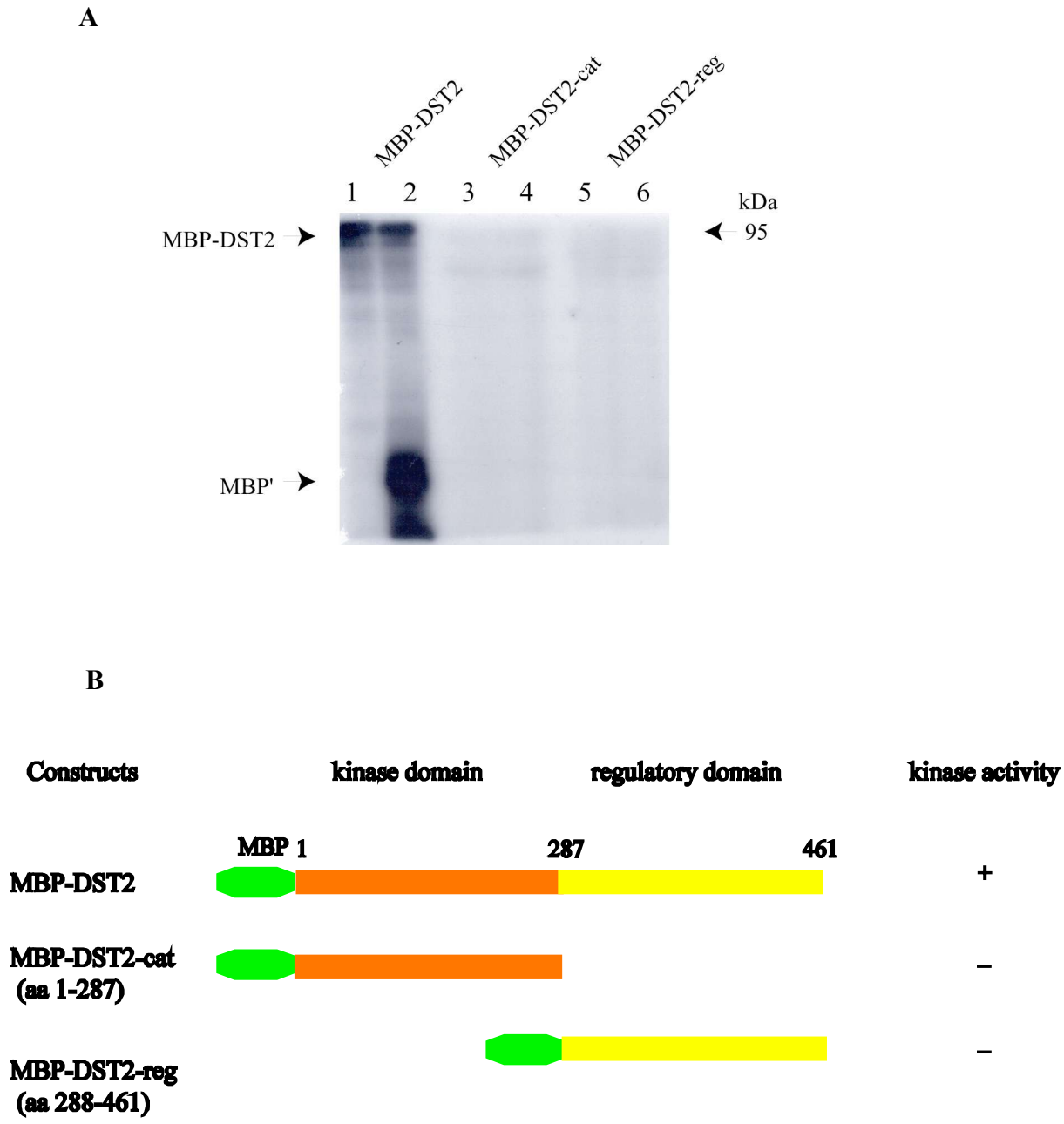


Fig. 11 *In vitro* kinase assays with recombinant MBP-DST2, MBP-DST2-cat and MBP-DST2-reg in the presence or absence of MBP'.
A: Purified recombinant MBP-DST2, MBP-DST2-cat and MBP-DST2-reg were subjected to an *in vitro* kinase assay alone (lanes 1, 3, 5) or with myelin basic Protein (MBP') (lanes 2, 4, 6). The positions of MBP-DST2 and MBP' are indicated. B: The corresponding DST2 constructs are schematically depicted.

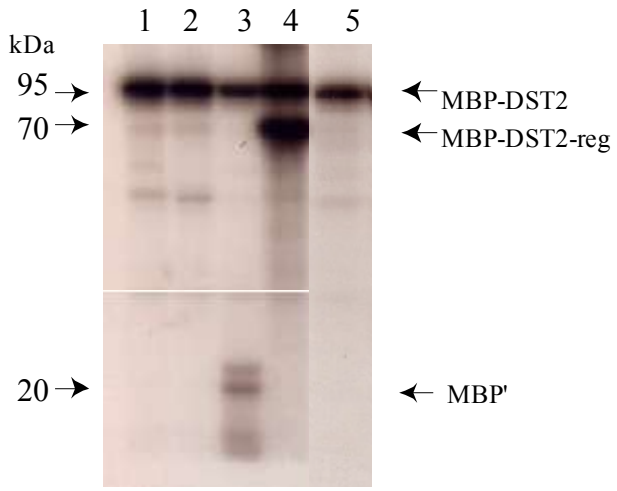


Fig. 12 *In vitro* kinase assays with MBP-DST2 alone or in the presence of MBP, MBP', MBP-DST2-reg or MBP-DST2-cat.
Full length MBP-DST2 was subjected to the *in vitro* kinase assay alone (1), or in the presence of either maltose binding protein (MBP, 2), myelin basic protein (MBP', 3), MBP-DST2-reg (4) or MBP-DST2-cat (5) as substrates. The positions of MBP-DST2, MBP-DST2-reg and MBP' are indicated on the right and sizes for molecular mass markers on the left.

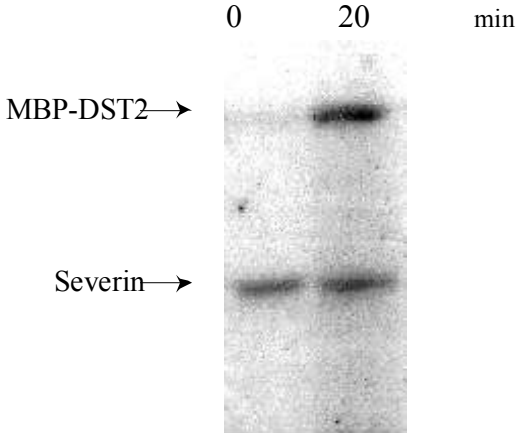


Fig. 13 The influence of autophosphorylation on the catalytic activity of MBP-DST2.
MBP-DST2 was incubated in the presence of ^{32}P-ATP for 0 or 20 min at 30°C followed by the addition of 1μg severin for an additional 5 min. The reaction products were separated by SDS-PAGE and subjected to autoradiography. The positions of MBP-DST2 and severin are indicated.

3.2.3.1 The influence of Mn^{2+} and Mg^{2+} on DST2 kinase activity

Recently, Schinkmann and Blenis reported that mammalian STE20-like kinase 3, MST3, prefers Mn^{2+} over Mg^{2+} as a divalent cation. In *in vitro* kinase assays they showed that the catalytic activity of MST3 was consistently 20-50 fold higher in the presence of Mn^{2+} as compared with Mg^{2+}, whereas MST3 activity did not change upon raising the intercellular Ca^{2+} or cAMP concentration (Schinkmann and Blenis, 1997). Mn^{2+} is also the most potent divalent cation activator of autophosphorylation for the tyrosine kinases of the insulin and epidermal growth factor receptors (White *et al*., 1984; Carpenter *et al*., 1978).

To determine the role of Mn^{2+} versus Mg^{2+} for DST2, MBP-DST2 activity was examined using either Mg^{2+} or Mn^{2+} in an *in vitro* kinase assay with either MBP' or severin as a substrate (Fig.14). Interestingly, under these conditions, MBP-DST2 catalytic activity was found to be much higher (~10 fold) in the presence of Mn^{2+} as compared to Mg^{2+}, suggesting that DST2 prefers Mn^{2+} as a cofactor. Similarly, the autophosphorylation activity was elevated in the presence of Mn^{2+}. The weaker bands seen in the assay with Mn^{2+} are degradation products of recombinant MBP-DST2.

To determine whether the kinase activity of DST2 is dependent on the Mn^{2+} concentrations, MBP-DST2 activity was tested using a range of Mn^{2+} concentrations. *In vitro* kinase assays were performed using the indicated Mn^{2+} concentrations with severin as a substrate. As shown in Fig.15, severin was phosphorylated strongly by MBP-DST2 with rising Mn^{2+} concentrations. In order to phosphorylate severin the Mn^{2+} concentration had to be at least 0.5mM and phosphorylation strongly increased with increasing Mn^{2+} concentration. Phosphorylated severin was cut out, eluted and subjected to mass spectrometry. The data indicate that severin contains several phosphorylation sites, which all seem to be located in the third domain, between residues 260-315 (data not shown). Interestingly, previous data showed that domain 2+3 of severin is also a substrate for DST1 (Eichinger *et al*., 1998).

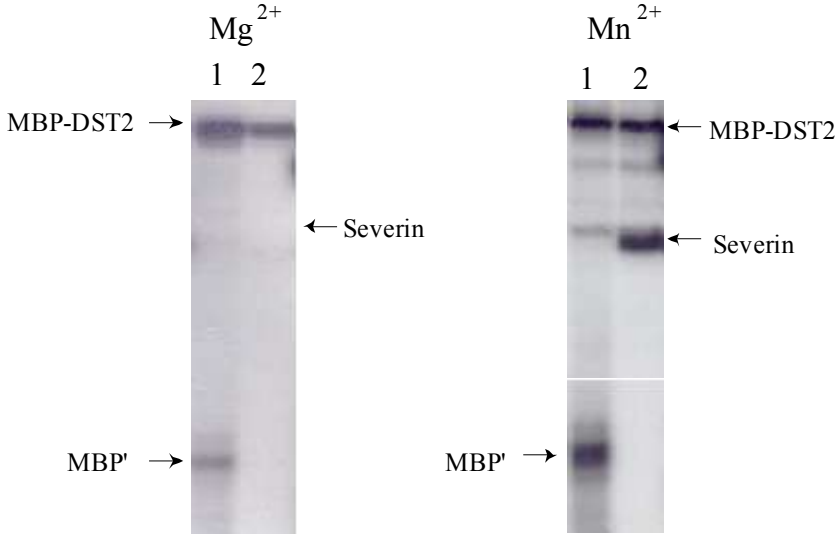


Fig. 14 Regulation of MBP-DST2 kinase activity by divalent cations.
MBP-DST2 was used in the *in vitro* kinase assay with MBP'(lane 1) or severin (lane2) as substrates in the presence of Mn^{2+} (10mM) or Mg^{2+} (10mM). The reactions were subjected to SDS-PAGE and visualized by autoradiography. Positions of DST2, MBP' and severin are indicated.

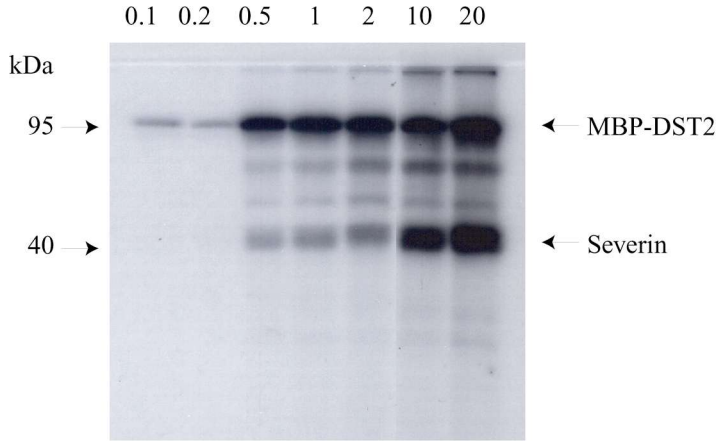


Fig. 15 MBP-DST2 kinase activity in the presence of increasing concentrations of Mn^{2+}.
MBP-DST2 was used in the *in vitro* kinase assay with different concentrations of Mn^{2+} using severin as a substrate. The Mn^{2+} concentration is indicated in mM on top and the position of molecular mass markers on the left. Positions of DST2 and severin are indicated.

3.2.3.2 PKA is a potential upstream kinase of DST2

Protein kinase A (PKA) is a mediator of the actions of hormones and neurotransmitters that activate adenylcyclase via heterotrimeric G-proteins thereby increasing the intracellular cyclic AMP level (Taylor *et al*., 1990; Walsh and Van Patten, 1994). Recently, it has been reported, that MST3b, a human brain specific STE20-like kinase which activates the p42/44 MAPK signalling pathway, is negatively regulated by PKA (Zhou *et al*., 2000). This result suggested that PKA might act as a potential upstream kinase for the GCK subfamily of STE20-like protein kinases. To address the question whether PKA can phosphorylate and regulate DST2, MBP-DST2, MBP-DST2-reg and PKA were subjected to *in vitro* kinase assays alone and in different combinations in the presence of either 10mM Mg^{2+} or 10mM Mn^{2+} (Fig. 16). In the presence of 10mM Mn^{2+}, the addition of PKA induced a strong increase in the basal autophosphorylation activity of MBP-DST2 (Fig. 16B, lanes 1, 2). No increase in activity was seen in the presence of Mg^{2+} (Fig. 16A, lanes 1, 2). PKA showed autophosphorylation activity and the recombinant regulatory domain of DST2, MBP-DST2-reg, was effectively phosphorylated by PKA (Fig. 16 A, B, lanes 3, 4). MBP-DST2-reg alone was not phosphorylated (data not shown). This result suggested that PKA may be a potential upstream regulating kinase for DST2 through the phosphorylation of its regulatory domain.

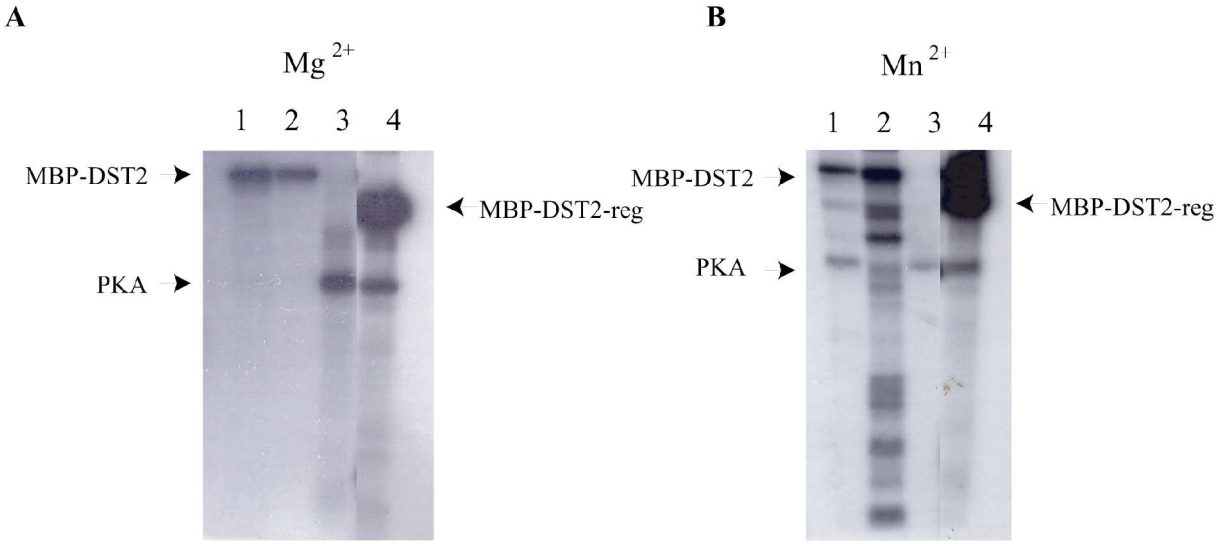


Fig. 16 Phosphorylation of DST2 by PKA.
In vitro kinase assay were carried out either in the presence of 10mM Mg^{2+} (A) or in the presence of 10mM Mn^{2+} (B). MBP-DST2 was either subjected to phosphorylation alone (lanes 1) or in the presence of purified catalytic subunit of PKA (lanes 2). The catalytic subunit of PKA was either phosphorylated alone (lanes 3), or in the presence of the regulatory domain of DST2, MBP-DST2-reg (lanes 4). The positions of MBP-DST2, PKA and MBP-DST2-reg are indicated.

3.2.4 Expression, purification and biochemical characterisation of recombinant C-terminally truncated DST2 constructs

The catalytic domain of DST2 is highly homologous with the kinase domains of the GCK subfamily. However, the putative regulatory domain of DST2 has no significant similarities to other known proteins. To understand its function, C-terminally truncated constructs were created. In addition to the previously described construct MBP-DST2-cat (see Fig. 11), the catalytic domain of DST2, we generated three additional deletion constructs within the DST2 carboxyl terminus by PCR. These code for MBP-DST2(1-421), MBP-DST2(1-368) and MBP-DST2(1-326) and end at the indicated amino acid of DST2. The corresponding products were expressed in *E. coli* and purified as maltose binding proteins (see 2.4.7.2). Their purification is shown in Fig. 17. Immunoblots with an antibody against the regulatory domain of DST2 showed that most of the additional bands in the eluted fractions are degradation products (data not shown).

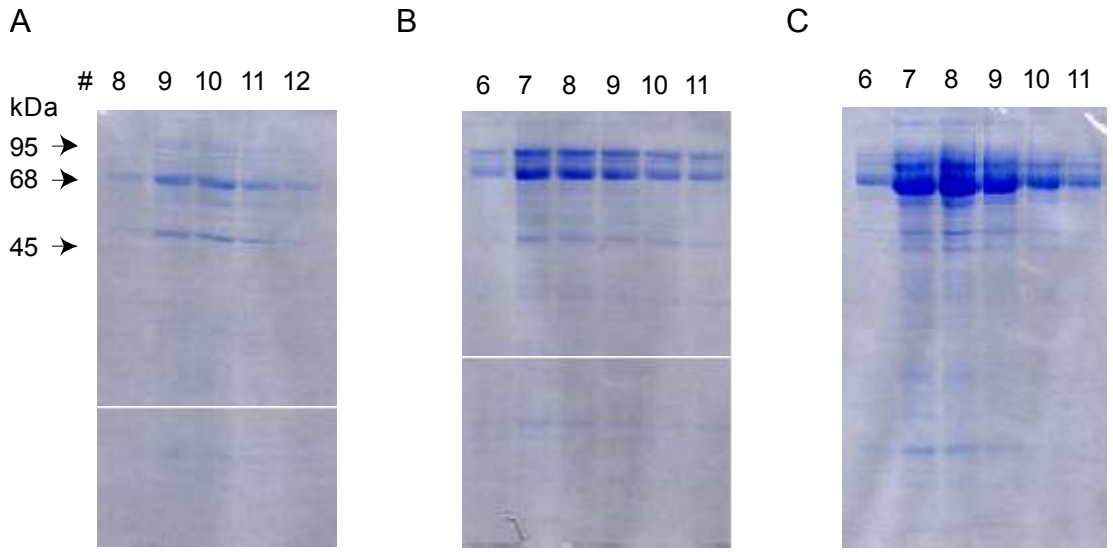
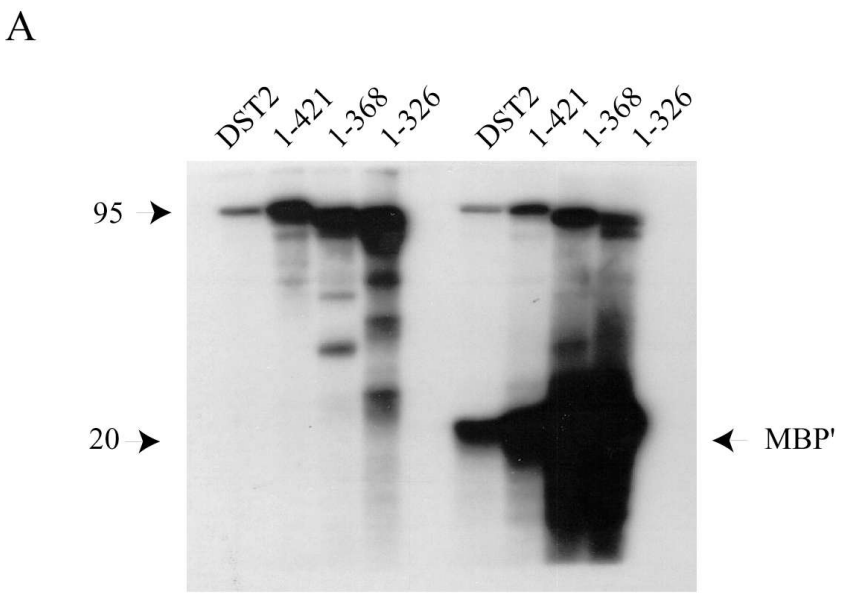


Fig. 17 Purification of C-terminally truncated constructs of DST2.
Three C-terminally truncated constructs were generated via PCR, inserted into the pMal-vector, and expressed. The recombinant DST2 deletion constructs were purified by affinity chromatography with an amylose resin. A: MBP-DST2(1-421), B: MBP-DST2(1-368), C: MBP-DST2(1-326). The positions of the purified constructs are indicated by an arrow. The fraction size was 0.5 ml and the fraction numbers are give above the gel. The positions of the molecular mass markers are indicated on the left.

3.2.5.1 *In vitro* kinase assays with C-terminally truncated constructs of DST2

For MST1, MST2 and SOK-1, all members of the GCK subfamily, it was shown that the putative regulatory domain apparently has an inhibitory function, because its removal resulted in an increase in protein kinase activity (Pombo *et al*., 1996; Creasy *et al*., 1996). Since MST1 is the protein kinase with the highest homology to DST2 we decided to map the potential inhibitory region of DST2 and generated three C-terminal deletion constructs, MBP-DST2 (1-421), MBP-DST2 (1-368) and MBP-DST2 (1-326). These were used in comparison to full length DST2 (MBP-DST2) in an *in vitro* kinase assay with or without MBP' as a substrate. MBP-DST2 (1-421), the deletion construct lacking the last 40 amino acids of DST2 showed an app. 2 fold increase in kinase activity. A further deletion of an additional 53 amino acids in MBP-DST2(1-368) resulted in an app. 10 fold increase in kinase activity, and a deletion of 42 amino acids more MBP-DST2(1-326) increased the activity by another factor of 5. We also detected an increase in the autophosphorylation levels of the truncated constructs. This result suggests that within amino acids 326-461 of DST2 an inhibitory region is present that negatively regulates its kinase activity (Fig. 18). Surprisingly, deletion of the complete regulatory domain (MBP-DST2-cat, comprising aa 1-287 of DST2) resulted in a complete loss of catalytic activity (see Fig. 11). This could be either due to folding problems or, alternatively, part of the regulatory domain is needed for the catalytic activity of DST2.



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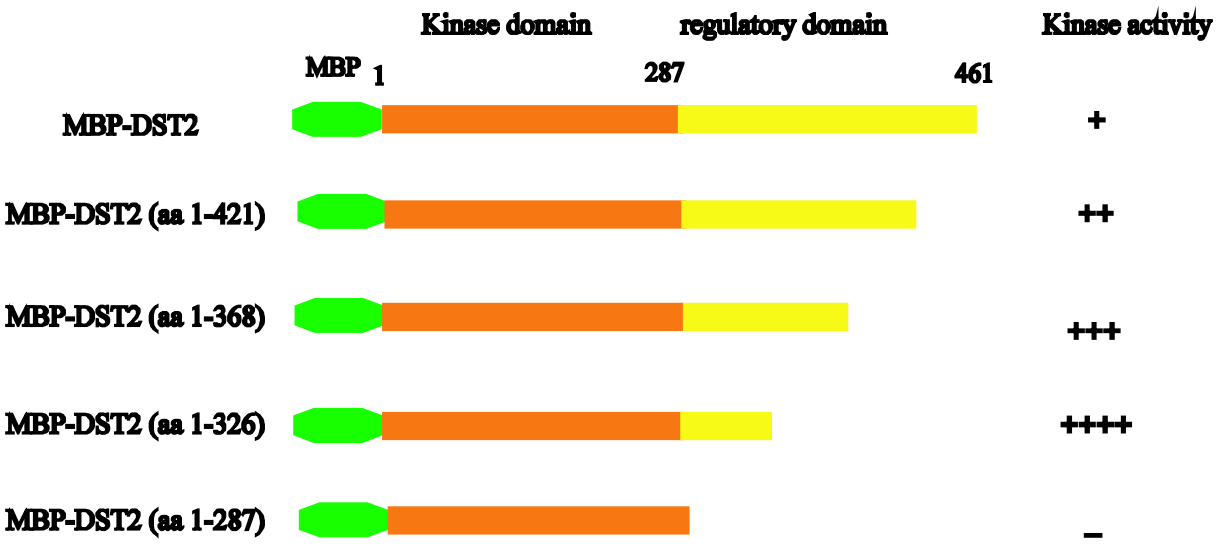

Fig. 18 The C-terminal regulatory domain of DST2 inhibits its catalytic activity.
A: Three C-terminal deletion constructs, MBP-DST2(1-421), MBP-DST2(1-368), MBP-DST2(1-326) were used in comparison to full length DST2 (MBP-DST2) in an *in vitro* kinase assay with (right four lanes) or without (left four lanes) MBP' as a substrate. The identities of the constructs used in the kinase assay are indicated above the gel, the positions of the molecular mass markers is given on the left and the position of MBP' on the right. B: Schematic presentation of full length MBP-tagged DST2 and the deletion constructs. The corresponding catalytic activity is indicated on the right. The catalytic domain of DST2 (MBP-DST2-cat ; aa1-287) did not show kinase activity (see Fig. 11).

3.2.6 DST2 is present as a high molecular weight complex in AX2 homogenate

It has been suggested that members of the GCK family may oligomerize (Pombo *et al.*, 1995). Sequence comparisons of the regulatory domain of DST2 with the corresponding domain of the other kinases in the GCK family did not reveal significant sequence similarity to most of these kinases. MST1, which dimerizes or may exist as a multimer (Creasy *et al.*, 1996), was the only family member, which displayed some sequence similarity with the regulatory domain of DST2.

Gel filtration chromatography was used to investigate whether endogenous DST2 may exist as a high molecular weight complex. The soluble fraction of wild type *Dictyostelium* cells (100,000xg supernatant) was subjected to gel filtration chromatography using a Superose 12 column. The eluted fractions were analyzed by immunoblotting with a polyclonal antiserum raised against the regulatory domain of DST2.

DST2 was eluted in a broad peak ranging from approximately 63 kDa to 400 kDa, suggesting that DST2 may exist *in vivo* as a monomer as well as a high molecular weight complex (Fig. 19). The composition of this complex is not clear at present.

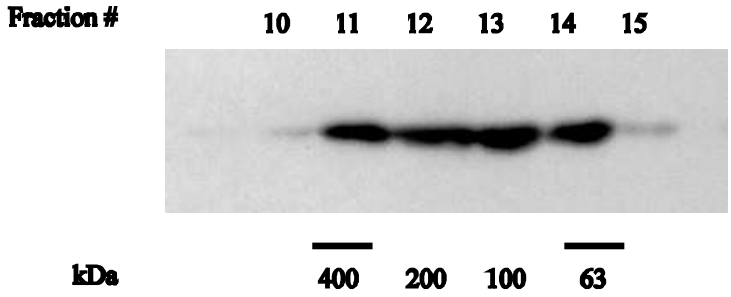

Fig. 19 Endogenous DST2 exists in a high molecular weight complex.
D. discoideum cells were lysed, total cell homogenate was centrifuged at 100,000xg and the soluble fraction subjected to gel filtration chromatography using a Superose 12 column. Fractions were analyzed by immunoblotting with a polyclonal antiserum against the regulatory domain of DST2. The positions of the calibration markers and the fraction numbers are indicated.

3.2.7 Phosphorylation by DST2 inhibits the severing activity of severin

We found that severin was phosphorylated by DST2 in Mn^{2+} dependent manner. However, it was not clear, whether phosphorylation regulates the activity of severin. To address this question, the influence of phosphorylated and unphosphorylated severin on F-actin solutions was investigated using falling-ball viscometry (see 2.4.8) and fluorescence spectroscopy (see 2.4.9). The recombinant construct MBP-DST2 (1-326), which showed the highest catalytic activity, was used in these assays. In the low shear falling-ball viscometry assay, severin phosphorylated by DST2 did not decrease the viscosity of F-actin, whereas unphosphorylated severin was found to reduce the viscosity as expected (data not shown). We also measured the fluorescence decrease of pyrene-labeled F-actin in a dilution induced depolymerisation assay in the presence of either phosphorylated or unphosphorylated severin. In this assay F-actin fragmenting proteins create additional pointed ends which results in a faster depolymerisation of the pyren-labeled F-actin.

We found that increasing concentrations of unphosphorylated severin resulted in an increasing decrease in fluorescence while identical concentrations of phosphorylated severin only moderately influenced the fluorescence decrease in comparison to the control (Fig. 20). This result strongly suggested that phosphorylation by DST2 inhibits the F-actin fragmenting activity of severin.

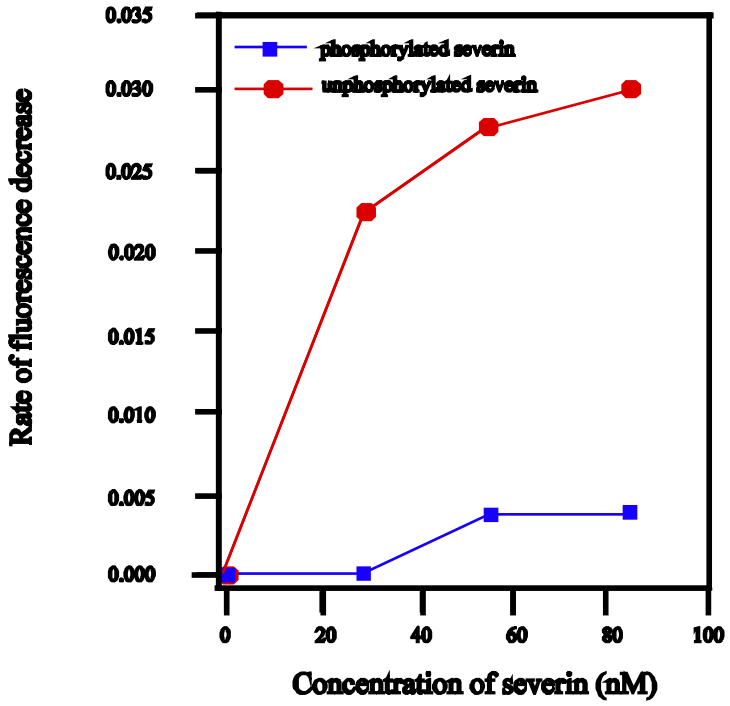

Fig. 20 Dilution induced depolymerisation of pyrene-lebaled F-actin in the presence of phosphorylated or unphosphorylated severin.
Severin was incubated with or without MBP-DST2 (1-326) in kinase buffer for 20 min at room temperature. These samples were then used in the dilution induced depolymerisation assay with pyrene-labeled F-actin. The rate of fluorescence decrease of pyrene-labeled F-actin with phosphorylated or unphosphorylated severin was measured. The measured relative fluorescence values were plotted against time and the slopes of these plots were calculated and plotted versus severin concentration.

Chapter 4

Discussion

4.1 DST2 is a new member of the STE20-like protein kinase family

We screened the sequences of the *D. discoideum* cDNA project database in Tsukuba, Japan (Tsukuba website: http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html), using DST1 (*Dictyostelium* STE20-like kinase 1) as query and discovered a cDNA clone coding for a highly homologous protein kinase. Pending the identification of a physiological function, this kinase was named DST2 (*Dictyostelium* STE20-like kinase 2). The 1.5 kilobase cDNA insert contained an open reading frame coding for a protein of 461 amino acids with a predicted molecular mass of 52 kDa. Based on sequence homologies, DST2 was identified as a new member of the GCK subfamily of STE20-like kinases with an N-terminal catalytic 287-amino acids and a C-terminal regulatory domain 174-amino acids. DST2 displays 69% amino acid similarity to the kinase domain of DST1. DST1 is activated by osmotic stress, phosphorylates the F-actin fragmenting protein severin and is most closely related to human SOK1, which is activated by oxidant stress (Pombo *et al*., 1996; Eichinger *et al*., 1998; unpublished results). Further sequence comparisons showed that DST2 is most closely related to human MST1 with 79% amino acid similarity in the kinase.

The strong similarity of DST2 and MST1 over their entire length suggests that these two protein kinases are orthologues and might have similar or identical *in vivo* function. MST1 dimerizes and exists in a high molecular weight complex. Its catalytic activity is inhibited by the regulatory domain. MST1 is activated upon treatment of cells with staurosporine, okadaic acid, high concentrations of sodium arsenite, and extreme heat shock at 55°C (Creasy and Chernoff, 1995; Creasy *et al*., 1996; Taylor *et al.*, 1996). Furthermore, it was found that MST1 is specifically cleaved by a caspase-3 like activity during apoptosis, induced by CD95/Fas. Mutational analysis indicated that the caspase recognition and cleavage site in MST1 is DEMD, which is similar to the consensus sequence for caspase 3 (DEVD) (Graves *et al*., 1998). Interestingly, DST2 contains the sequence motif DEQD at the border between the N-terminal kinase domain and the C-terminal regulatory domain (Fig. 21).

Little is known about apoptosis in *Dictyostelium* and it remains to be determined, whether DST2 could be a subject for specific proteolysis in this process.

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DST2(Dd)     ~~~~~~~~~~   ~~~~~~MSTL   NVPKETMSRK   DPEKFFTIVE   KLGEGSYGSV
MST1(HS)     ~~~~~~METV   QLRNPPRRQL   KKLDEDSLTK   QPEEVFDVLE   KLGEGSYGSV
                                  *          *        **  *  **   **********
DST2(Dd)     YKAINISTGI   VVAIKKVSVD   NDLEDMEKEI   SFMKQCKSPY   IVTYYASFRK
MST1(Hs)     YKAIHKETGQ   IVAIKQVPVE   SDLQEIIKEI   SIMQQCDSPH   VVKYYGSYFK
             ****   **    **** ***     * ** ***   * * ** **    * ** *  *
DST2(Dd)     ENEVWIVMEH   CGAGSVCDAM   KITDKTLSED   QIAVVSRDVL   QGLAYLHSVR
MST1(Hs)     NTDLWIVMEY   CGAGSVSDII   RLRNKTLTED   EIATILQSTL   KGLEYLHFMR
                 *****    ****** *      *  *** **    **       *    ** ***  *
DST2(Dd)     KIHRDIKAGN   ILMNHKGESK   LADFGVSGQL   SDTMAKRQTV   IGTPFWMAPE
MST1(Hs)     KIHRDIKAGN   ILLNTEGHAK   LADFGVAGQL   TDTMAKRNTV   IGTPFWMAPE
             **********   ** *  *  *   ****** ***    ****** **   **********
DST2(Dd)     VIQEIGYDYK   ADIWSYGITC   IEMAESKPPL   FNVHPMRVIF   MIPNPSRPPP
MST1(Hs)     VIQEIGYNCV   ADIWSLGITA   IEMAEGKPPY   ADIHPMRAIF   MI..PTNPPP
             *******      ***** ***    ***** ***      **** **   **  *  ***
DST2(Dd)     KLTEPEKWSP   EFNDFLAKCL   TRKPELRPSA   EELLKHPFIT   KAKSHSLLVP
MST1(Hs)     TFRKPELWSD   NFTDFVKQCL   VKSPEQRATA   TQLLQHPFVR   SAKGVSILRD
                 ** **     * **   **     **    *     ** ***      **  ***
DST2(Dd)     LIDEQDIIIN   EKGREVALGI   EQRDEEEEDE   DEDSEDSDDN   RGTMVRAKPR
MST1(Hs)     LINEA.MDVK   LKRQE.....   SQQREVDQDD   EENSEEDEMD   SGTMVRAVGD
             ** *          *  *         *  *   *     * **         ******
DST2(Dd)     SMQNSGGEDN   DEEYDTGTMV   ITDN..KNSY   DTVVFNNDD.   .EDSGTMKLK
MST1(Hs)     EMGTVRVAST   MTD.GANTMI   EHDDTLPSQL   GTMVINAEDE   EEE.GTMKRR
              *                  **     *           * * *  *     *  ****
DST2(Dd)     NTMPSNKKNF   VPDYMNQFKK   SDDDVTN...   ..........   .... VPLSD
MST1(Hs)     DETMQPAKPS   FLEYFEQ.KE   KENQINSFGK   SVPGPLKN..   SSDWKIPQDG
                    *          * *
DST2(Dd)     KY...SSYSL   EELKKMLAEL   EIEREKEVQK   TLEKFSINRQ   ALLAVIDEKK
MST1(Hs)     DYEFLKSWTV   EDLQKRLLAL   DPMMEQEIEE   IRQKYQSKRQ   PILDAIEAKK
              *    *      * * * *  *       * *              **   **  *  **
DST2(Dd)     SK
MST1(Hs)     RRQQNF
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Fig. 21. Sequence alignment of *Dictyostelium* DST2 and human MST1.
The sequence alignment was done with the program Clustal from UWGCG program package. Identical residues are indicated by a star. Pseudo-consensus sequence for caspase 3 DEVD in MST1 and DEQD in DST2 are shaded in grey.

4.2. DST2 is autophosphorylated and activated in Mn^{2+} dependent manner

We used MBP-tagged recombinant DST2 in *in vitro* kinase assays and found that DST2 possesses unusual cofactor requirements. The ability to phosphorylate itself and exogenous substrates was consistently more than 10-fold higher in the presence of Mn^{2+} *versus* Mg^{2+}. The increase of DST2 activity observed in response to increasing Mn^{2+} concentration suggested that besides formation of the substrate MnATP, also binding of free Mn^{2+} to a distinct site on DST2 might be required for full kinase activity. Mn^{2+} is the most potent divalent cation activator of autophosphorylation for the tyrosine kinases of the insulin and epidermal growth factor receptors (White *et al.*, 1984; Carpenter *et al.*, 1978). Recently, it was shown that mammalian STE20-like kinase 3 (MST3), a close homologue of MST1, MST2 and DST2, also prefers Mn^{2+} to Mg^{2+} as a cofactor. MST3 catalytic activity was found to be 20-50 fold higher in the presence of Mn^{2+} compared with Mg^{2+} (Schinkmann *et al.*, 1997). However, the physiological role of Mn^{2+} in the activation of DST2 and MST3 needs to be elucidated.

4.3 Regulation of DST2 activity

The protein kinase activity of PAKs can be stimulated by binding of activated GTP-bound Cdc42 and Rac (Manser *et al.*, 1994). Accumulated data suggest that PAK kinase activity is repressed by an intramolecular interaction between the regulatory and catalytic domains. Binding of GTP-bound Cdc42 or Rac disrupts this interaction resulting in a stimulation of kinase activity (Bagrodia and Cerione, 1999).

For members of the GCK subfamily the putative regulatory role of the C-terminal non-catalytic domain is not clear. In the case of MST1, MST2, and SOK-1 it apparently has an inhibitory function because its removal resulted in an increase in kinase activity (Pombo *et al.*, 1996; Creasy *et al.*, 1996). Furthermore, it has been shown that the C-terminal domains of MST1 and MST2 mediate homo- and heterodimerization (Creasy *et al.*, 1996). In order to investigate the role of the putative regulatory domain of DST2 we performed a series of C-terminal deletions and also looked for potential upstream effectors. The C-terminal deletion results showed that DST2 contains a region between amino acids 326-461 that inhibits its catalytic activity (3.2.5).

There are several possible mechanisms by which this region may regulate kinase activity. Firstly, an inhibitory protein may bind to this region, as has been suggested for the related GCK (Pombo *et al*., 1995). Inactivation of the inhibitory molecule would in turn activate the kinase. Our *in vitro* kinase assay results argue against this scenario. Secondly, similar to the PAKs, the C-terminal domain per se might inhibit the catalytic activity. Our results support this model since several C-terminal deletions of DST2 were found to be much more active than the full length molecule (Fig. 22). If this model is correct we are faced with the question how the inhibitory role of the regulatory domain might be overcome *in vivo*. One possibility is regulation by autophosphorylation and in *in vitro* kinase assays we found that the regulatory domain was phosphorylated by full length DST2, suggesting that there might be a pseudosubstrate site within this domain. However, no change in activity was observed. Another possibility is activation through an upstream effector. We found that the regulatory domain contains several potential phosphorylation sites for PKA, it was phosphorylated by PKA *in vitro* and phosphorylation of recombinant DST2 by PKA significantly increased its activity (3.2.3.2). Taken together, these data support a model, in which phosphorylation of the regulatory domain by either PKA or another as yet unknown protein kinase, triggers a conformational change that leads to activation of DST2.

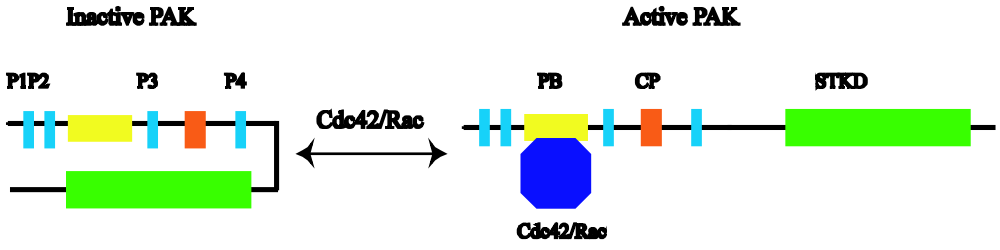

Fig. 22 Schematic representation of human PAK1 regulation.
Depiction of the Cdc42/Rac stimulated activation of PAK. The binding of Cdc42/Rac reverses an autoinhibitory intramolecular interaction. The following domains or sequence motifs are indicated. P1, P2, P3, P4: potential SH3 (Src-homology 3) binding motifs; PB: P21-binding domain; CP: cool-pix-binding region; STDK: serine-threonine kinase domain (adapted from Bagrodia and Cerione, 1999).

4.4. DST2 exists as a high molecular weight complex

In gel filtration chromatography (3.2.6), DST2 was eluted in a broad peak ranging from approximately 63 kDa to 400 kDa, suggesting that DST2 may be part of a high molecular weight complex *in vivo*. However, the composition of this complex is not clear at all. It could be either composed of DST2 only or of DST2 plus additional proteins. Human MST1, which is the closest homologue to DST2 and the only group II GCK subfamily member with similarity to the regulatory domain of DST2, homodimerizes and seems to be associated with additional proteins (Creasy *et al*., 1996). Therefore, we favor a model in which also in *D. discoideum* a DST2 dimer is associated with additional proteins. Purification of native DST2 kinase from *D. discoideum* should give insight into the composition and the stoichiometry of the proposed complex. The functional significance of complex formation of MST1 and possibly DST2 and other kinases of the GCK subfamily is not clear. For some protein kinases dimerization seems to be important for recognition by an effector molecule or phosphorylation specificity. One such example is the monomeric cGMP-dependent protein kinase, which phosphorylated histone and peptides, whereas only the dimer phosphorylated vimentin, the protein suspected to be the real *in vivo* substrate (MacMillan-Crow and Lincoln, 1994).

4.5. Binding partners of PAK family kinases

Recently, several PAK-binding proteins were identified. These PAK binding partners are p50Cool-1, p85Cool-1/βPix, Cool-2/αPix, which contain SH3 (src-homology), DH (Dbl-homology) and PH (Pleckstrin-homology) domains (Manser *et al*., 1998). The binding of Cool/Pix to PAK seems to activate PAK either by cooperating with the binding of activated Cdc42 or Rac or by altering PAK confirmation (Bagrodia and Cerione, 1999).
Some of the group I GCKs might be downstream effectors of TNF (tumor necrosis factor). Binding of TNF to the TNF receptor recruits TRAF2 (TNF receptor-associated factor 2) and it was found that GCK and GCLK can be associated *in vivo* with TRAF2. This result suggested that GCK and GCKR may be important effectors for TRAF2 (Pombo *et al*., 1995; Yuasa *et al*., 1998; Arch *et al*., 1998). So far no binding partners have been identified for the Group II GCK subfamily, to which DST2 belongs to.

However, it was found that the Group II GCK subfamily can be activated by different environmental stresses. MST1 and MST2 can be activated by treatment of cells with staurosporine, okadaic acid, high concentrations of sodium arsenite, and extreme heat shock at 55°C (Creasy *et al*., 1996). MST1 could also be activated *in vitro* by phosphatase 2A suggesting that MST1 might be activated by dephosphorylation (Taylor *et al*., 1996). SOK1 is strongly activated by oxidant stress and also by ischemic injury (Pombo *et al*., 1996; Schinkmann and Blenis, 1997). Nothing is known about environmental conditions that might trigger activation of DST2. However, its pronounced similarity to human MST1 suggests a possible role of DST2 in an as yet unknown stress response pathway in *D. discoideum*. Knock-out experiments as well as treatment of cells with various stresses in combination with *in vivo* labelling and immunoprecipitation should provide insight into the physiological role of DST2.

4.6. Signalling to the cytoskeleton

Several members of the PAK family have recently been implicated in cytoskeletal reorganization or the regulation of cytoskeletal proteins. PAK1 kinase activity is essential for disassembly of focal adhesion (Frost *et al*., 1998). LIMK1 (LIM-motif-containing protein kinase) phosphorylates the actin-depolymerizing factor cofilin and serves as a target for PAK1 (Edwards *et al*.,1999). MIHCK from *Dictyostelium* and its homologue from *Acanthamoeba* phosphorylate the heavy chain of some of the myosinI isozymes on a single serine or threonine residue and thereby stimulate their actin-activated MgATPase activity 30 to 50 fold (Lee and Côté, 1995; Brzeska and Korn, 1996). Cloning of the corresponding genes revealed that MIHCK is a member of the PAK family and closely related to mammalian PAK and yeast Ste20p molecules (Lee *et al*., 1996; Brzeska *et al*., 1996). In gel overlay assays and affinity chromatography experiments MIHCK from *Dictyostelium* interacted with GTPγS-labeled Rac1 and Cdc42, which probably bind to a conserved PBD commonly found in the N-terminal regulatory domain of true PAKs. Interestingly, in the presence of active Rac1 and Cdc42 autophosphorylation of MIHCK increased from 1 up to 9 moles of phosphate per mol of kinase concomitant with an approximately 10-fold stimulation of the rate of myosin ID phosphorylation. These results suggest that MIHCK directly links Cdc42/Rac signalling pathways to motile processes driven by myosin I molecules (Lee *et al*., 1996).

The role of GCK subfamily members in signalling to the cytoskeleton is less clear. Recently it was shown that overexpression of either wild type MST1 or a truncated mutant induced morphological changes (Graves *et al*., 1998). DST1 was found to phosphorylate the actin fragmenting protein severin (Eichinger *et al*., 1998). *In vitro* TNIK could phosphorylate gelsolin, the mammalian severin homologue and overexpression of wild type TNIK resulted in the disruption of F-actin structure and the inhibition of cell spreading. (Fu *et al*., 1999).

Here we showed that native severin was also a substrate for DST2 *in vitro* (3.2.3.1). Severin is a Ca^{2+}- activated actin-binding protein that nucleates actin assembly, fragments F-actin and caps the newly created barbed end. It consists of three highly conserved domains. Domain 1 of severin is responsible for F-actin capping, domains 1/2 cooperate in F-actin fragmenting whereas domains 2/3 possess two F-actin binding sites (Eichinger e*t al*., 1992). Phosphorylation of severin by DST2 resulted in an inhibition of severin's F-actin fragmenting activity (see 3.2.7), suggesting that DST2 may play a role in the remodeling of the actin cytoskeleton. Mass spectrometry data suggested that all phosphorylation sites on severin seem to be in the third domain, between residues 260-315. Phoshorylation of domain 3 might lead to a conformational change and thus inhibit the F-actin fragmenting activity of severin.

Several recent findings indicate that PKA functions in regulating STE20-like kinases. Functional connections between PKA and PAKs have been demonstrated in the regulation of oocyte maturation in *Xenopus* and chemotaxis in *Dictyostelium* (Faure *et al*., 1999; Chung *et al*., 1999). MST3b was directly phosphorylated and inhibited by PKA *in vivo* and *in vitro* (Zhou *et al*., 2000). We have shown (3.2.3.2) that exposure of DST2 in *in vitro* kinase assays to the purified catalytic subunit of PKA resulted in the phosphorylation of the regulatory domain and we also observed a strong increase in the basal autophosphorylation activity of DST2.

Taken together our data suggest that PKA might be a potential upstream regulatory kinase for DST2 which in turn might phosphorylate and regulate the F-actin fragmenting protein severin. The inhibition of severin's F-actin fragmenting activity might play a role in the reorganization of the actin cytoskeleton. Further studies are needed in order to unravel upstream and downstream components of signalling via DST2.

4.7. Future prospects

To learn more about the *in vivo* function of DST2, the following studies need to be done in the future:

1. Disruption of the *Dictyostelium* DST2 gene by homologous recombination.
2. Identification of possible binding partners of DST2 using the yeast two-hybrid system.
3. Expression of GFP-DST2 fusion proteins to investigate the intracellular localisation.
4. Production of better polyclonal and/or monoclonal antibodies against DST2.
5. Purification and characterisation of endogenous DST2 using affinity chromatography.
6. *In vivo* labelling experiments with cells treated with various stresses followed by immunoprecipitation of DST2 and/or severin.

Chapter 5

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