

<b>Background and Significance</b> .....	6
<b>The Myosin Superfamily</b> .....	6
<b>Domain Structure of Myosins</b> .....	7
<b>Myosin Crystal Structure</b> .....	9
<b>Myosin Regulation</b> .....	11
Myosin Regulation via Phosphorylation.....	12
Myosin Regulation via Calcium Binding.....	14
<b>ATPase Activity of Myosins</b> .....	14
<b>Processivity and Duty Ratio of Myosins</b> .....	15
<b>In vitro Motility Assays</b> .....	17
Single Molecule <i>in vitro</i> Assays.....	17
<b>Myosin V and Myosin VI as Processive Motors</b> .....	20
<b>Myosin VI is a Point-end Directed Motor</b> .....	23
Roles of Myosin VI as a Point-end Directed Motor in Cells.....	25
<b>Aims of the Project</b> .....	27
<b>Material and Methods (I)</b> .....	29
<b>Dissection of the Myosin VI Walking Mechanism</b> .....	29
Overview of the Cloning Strategy of KKCK- and CCRECC- tagged Calmodulin Gene.....	29
<b>Standard Molecular Biological Methods</b> .....	30
DNA Analysis Methods.....	30
DNA Purification and Isolation Protocols.....	30
Restriction Digest Reactions of DNA.....	31
Transformation of Chemically Competent <i>E. coli</i> DH5- $\alpha$ Cells.....	32
Protein Analysis Methods.....	32
Determination of the Protein Concentration.....	33
<b>Protein Expression in Baculovirus Expression System</b> .....	34
Advantages of the Baculovirus Protein Expression System.....	34
Protein Expression in the Baculovirus Expression System.....	35
Construction of the Recombinant Transfer Vector for Protein Expression -Step 1.....	35
Generation of the Recombinant Bacmid -Step 2 and Step3.....	36
Isolation of the Recombinant Bacmid and Conformation of Recombination via PCR -Step 4 and Step 5.....	37
Transfection of the SF-9 Cells with the Bacmid -Step 6.....	37
Amplification of the P <sub>1</sub> and P <sub>2</sub> Generations – Step 7 and Step 8.....	38
Protein Expression – Step 9.....	39

<b>PCR Mutagenesis and DNA Sequencing Primers</b> .....	39
Mutagenesis Primers for N-terminal Insertion of the KKCK- and CCRECC-tags into the Calmodulin Gene.....	39
DNA Sequencing Primers for Confirmation of the KKCK- and CCRECC Insertions.....	40
Primers for PCR Analysis of the Recombinant Bacmid .....	40
<b>PCR Reactions</b> .....	41
Insertion of the KKCK- and CCRECC-Tags into the p2Bac/pFastBac- wt-CaM Plasmid.....	41
PCR Analysis of the Recombinant Bacmid Plasmid.....	41
<b>Restriction Digest Reactions</b> .....	42
Analytical Restriction Digest Reactions.....	42
Preparative Restriction Digest Reactions .....	42
<b>Ligase Reactions</b> .....	43
<b>Transposition Protocol</b> .....	44
Creation of the Bacmid Plasmid in DH10Bac™ Cells.....	44
Isolation of Recombinant Bacmid DNA.....	45
<b>Transfection of Sf9 Cells with Recombinant Bacmid Plasmid</b> .....	46
Preparation of the Sf9 Cells for Transfection .....	46
Preparation of the Bacmid for Transfection .....	46
<b>Viral Amplification Protocols</b> .....	47
Procedure for Counting Sf9 Cells and Viability Determination.....	47
P <sub>1</sub> Viral Amplification .....	47
P <sub>2</sub> Viral Amplification .....	47
<b>Viral Titre Assays</b> .....	48
<b>FLAG-tagged Protein Purification and ReAsH Labeling of the CCRECC-tagged Myosin VI Construct</b> .....	49
<b>Protein Purification and Cy5 Dye Labeling of the KKCK-tagged Myosin VI Construct</b> .....	52
FLAG-tagged Protein Purification .....	52
Cy5 dye Labeling and Cation Exchange Chromatography.....	52
<b>In vitro Motility Assays</b> .....	53
Flow Cell Preparation .....	53
<b>Single Molecule In vitro Motility Assays</b> .....	55
Flow Cell Preparation .....	55
Suspension of Actin Filaments via 90 nm Spherical Beads .....	56
Actin Polymerization in the Flow Cell.....	57
Suspension of Actin Filaments via NEM-treated myosin II.....	57
Microscope Set Up .....	59

<b>Materials and Methods (II)</b> .....	61
<b>Single Molecule High Resolution Colocalization (SHREC)</b> .....	61
YFP Deletion in the p2Bac/pFastBac- <i>wt</i> -M5- <i>wt</i> -CaM Plasmid .....	61
Cy3 and Cy5 Labeled Calmodulin Exchange onto the YFP- deleted Myosin V Construct.....	64
<b>Single Molecule In vitro Motility Assays</b> .....	64
Flow Cell Preparation .....	65
<b>Materials and Methods (III)</b> .....	67
<b>Acetone Powder Preparation for G-actin Purification</b> .....	67
G-Actin Purification from Chicken Muscle Acetone Powder .....	68
<b>Insertion of the CCXXCC-motif into the wild type S1 Gene</b> .....	69
Site directed QuikChange <sup>®</sup> Mutagenesis and Primer Design .....	70
PCR Reaction to Introduce the CCXXCC-Motif .....	71
<b>Electroporation of Plasmids into AX3-Orf+ Cells</b> .....	73
<b>Culturing transformed Dictyostelium Orf+ Cell Lines</b> .....	74
<b>Myosin II S1 Protein Purification</b> .....	74
Myosin S1 Purification via (His) <sub>6</sub> tag .....	74
Myosin S1 Purification via Anion Exchange Chromatography .....	77
<b>RLC Phosphorylation via MLCK-A Kinase</b> .....	78
MLCK-A Protein Expression and Purification .....	78
RLC Kinasing Reaction .....	79
Urea-SDS-Glycerol Mini-Gel Preparation .....	80
<b>ATPase assays</b> .....	81
<b>Probing RLC and HC interaction via Cysteine Footprinting</b> .....	83
Cysteine Footprinting via NTCB Labeling .....	83
Cysteine Footprinting Protocol via ICAT Labeling .....	84
K339FIAsH tagged Kinesin Protein Expression and Purification .....	86
<b>Myosin II S1 Labeling with FIAsH-EDT<sub>2</sub></b> .....	87
<b>Nucleotide State dependent Crosslinking of RLC and HC</b> .....	88
<b>RESULTS AND DISCUSSION (I)</b> .....	89
<b>Dissection of the Myosin VI Walking Mechanism</b> .....	89
<b>Myosin VI Construct Design and Protein Expression</b> .....	90

Insertion of the KKCK- and the CCRECC-tags into the wild type Calmodulin .....	92
Generation of the Recombinant Bacmid Plasmid .....	93
Viral Titre Assays.....	93
FLAG-tagged Protein Purification of the CCRECC- and KKCK-tagged Myosin VI Constructs.....	94
Protein Purification of the KKCK-tagged and the wild type Myosin VI constructs via Ion Exchange Chromatography.....	94
ReAsH Labeling of the CCRECC-tagged Myosin VI Construct.....	95
Cy5 dye Labeling of the KKCK-tagged Myosin VI Construct .....	98
<i>In Vitro</i> motility of the N-terminally tagged myosin VI constructs.....	99
<b>Possible Stepping Models of Myosin VI</b> .....	100
<b>Myosin VI Walks on Suspended Actin Filaments</b> .....	103
<b>Fluorophores were localized with Nanometer Resolution</b> .....	106
<b>Myosin VI takes ~72 nm Steps on Actin</b> .....	109
<b>Stepping Mechanism of Myosin VI</b> .....	111
<b>RESULTS AND DISCUSSION (II)</b> .....	112
<b>Single molecule high resolution colocalization (SHREC)</b> .....	112
<b>Construct Design and Protein Expression</b> .....	113
Expression and Purification of the YFP-deleted Myosin V .....	114
Direct Demonstration of Hand-over-Hand Walking of Myosin V .....	115
<b>RESULTS AND DISCUSSION (III)</b> .....	117
<b>Crystal Structures of Myosin II Catalytic Domains Reveal a Large Rotation in the Converter Domain</b> .....	117
Protein Design and Purification .....	118
Wild type and Mutant Myosin II S1 Protein Purification .....	118
Actin Activated ATPase of the Mutant Myosin II S1 Proteins.....	119
FLAsH Labeling of the M-751 and M-724 Myosin II S1 Constructs.....	119
<b>The HC and the RLC can Interact in Solution</b> .....	121
<b>Characterization of the RLC-HC-Interaction</b> .....	123
Crosslinking Between RLC and HC Occurs Intramolecularly .....	123
Crosslinking of RLC and HC is Nucleotide State Dependent.....	124
Characterization of the Crosslinking Cysteines in RLC and HC via Cysteine Footprinting .....	125
<b>Myosin Light Chain Kinase-A Expression and Purification</b> .....	127
The RLC Phosphorylation Inhibits the RLC - HC Interaction.....	128

<b>Role of RLC Phosphorylation in Dictyostelium Myosin II</b> .....	129
<b>Zusammenfassung</b> .....	131
<b>Abstract</b> .....	133
<b>Literature</b> .....	135
<b>Tables</b> .....	142
<b>Overview of Cloned Plasmids</b> .....	142
<b>Overview of PCR Primers</b> .....	143
<b>Overview of Chemicals and their Sources</b> .....	145
<b>Overview of Enzymes and their Sources</b> .....	147
<b>Overview of Microorganisms</b> .....	149
<b>Overview of Columns and Resins for Protein Purification</b> .....	149
<b>Overview of the Kits</b> .....	149
<b>Molecular Structure of the Dyes</b> .....	150
<b>Abbreviations</b> .....	151
<b>Amino Acid Sequences of the Protein Constructs</b> .....	154
<b>Porcine Myosin VI Heavy Chain</b> .....	154
<b>Chicken Myosin V Heavy Chain</b> .....	154
<b>Dictyostelium discoideum Myosin II S1 Heavy Chain</b> .....	155
<b>Dictyostelium discoideum Essential Light Chain</b> .....	155
<b>Dictyostelium discoideum Regulatory Light Chain</b> .....	156
<b>Drosophila melanogaster Calmodulin</b> .....	156