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Title of the Ph.D. thesis- Structural, Functional and Biophysical Studies on Microtubule Affinity-Regulating Kinase 4: An Enzyme Plays Potential Role in Cell Division

MAP/Microtubule affinity-regulating kinase 4 (MARK4), the human ortholog of Par-1, belongs to the family Ser/Thr kinases. It is inseparably linked with many human diseases including various kind of cancer, diet induced obesity, type2 diabetes, and neurodegenerative disorders. we have cloned two variants of human Par-1d (MARK4), kinase domain (MARK4-F2), and kinase domain along with 59 N-terminal residues (MARK4-F1) in PQE30 vector. The recombinant MARK4 was expressed in the form of inclusion bodies in M15 cells. We used N-lauroyl sarcosine to purify our protein. Refolding was done simply by dialyzing proteins for 24 hrs-48 hrs by frequently changing buffers. The correct refolding of the recombinant protein was validated by ATPase assay. Our purification procedure is quick, simple and produces adequate quantity of proteins with high purity in a limited step.

Further, we have compared their (MARK4-F1 and MARK4-F2) stability at varying pH range. Structural and functional changes were observed by incubating both forms of MARK4 in buffers of different pH. We measured the secondary structure of MARK4 using circular diachroism and tertiary structure by measuring intrinsic fluorescence and absorbance properties, along with the size of proteins by dynamic light scattering. We observed that at extremes of pH (below pH 3.5 and above pH 9.0), MARK4 is quite stable. However, a remarkable aggregate formation was observed at intermediate pH (between pH 3.5 and 9.0). To further validate these results, we have modeled both forms of MARK4 and performed molecular dynamics simulation for 15 ns. The spectroscopic observations are in excellent agreement with the findings of molecular dynamics simulation.

Adding to this, we also demonstrated that both variants of MARK4 were phosphorylated by atypical PKC (aPKC) and their activities were increased in presence of increasing concentration of aPKC *in-vitro*. The phosphorylation was observed at the serine and threonine residues of MARK4. The interaction of MARK2 and MARK3 with aPKC and their negative regulation by aPKC is already known. This study confirms a functional link between aPKC and MARK, two central determinants of cell polarity, and it suggests that aPKC may regulate all four members of Par-1 through

phosphorylating them in polarized cells. We have also seen MARK4-F1 and MARK4-F2 ATPase hydrolysis in presence of each other. We observed that the activity of one variant was increased in presence of other variant.

To know the structural basis of the binding of ligands (inhibitors) or substrates with MARK4 and to design new kinase inhibitor derivatives as therapeutics against various linked diseases. Molecular docking studies revealed that the PKR-inhibitor binds in the large hydrophobic cavity of the kinase domain of MARK4 through several hydrophobic and hydrogen-bonded interactions and molecular dynamics simulation showed relatively stable parameters for the complex of MARK4-F1 and PKR-inhibitor than that of MARK4-F2. A significant decrease in the fluorescence intensity of MARK4 was observed on successive addition of the PKR-inhibitor. Using fluorescence data we have calculated the binding-affinity and the number of binding site for PKR-inhibitor to the MARK4. We also observed that 59 N-terminal residues of MARK4-F1 are helping in the binding of ligand which is further validated by a lower K_D value of MARK4-F1 for PKR-inhibitor as compared to MARK4-F2.

We took several well-known kinase inhibitors into account, docked them (using molecular docking) with MARK4 two variants MARK4-F1 and MARK4-F2 and sorted out those ligands which were showing best binding. Among them, three kinase inhibitors, BX-912, BX-795 and OTSSP167 (hyrochloride), were selected, and binding studies were performed using fluorescence spectroscopy and Molecular docking. Molecular dynamics simulations suggested that among these ligands, OTSSP167 (hyrochloride) was most promising and docking revealed that these ligands bind in the large hydrophobic cavity of the kinase domain of MARK4 through several hydrophobic and hydrogen bonded interactions. Moreover, 59 N-terminal residues of MARK4-F1 are helping in the binding of ligands.

Intrinsic fluorescence, significantly quenched by the addition of these ligands, studies also show lower K_D value of MARK4 with OTSSP167 (hyrochloride) suggesting it as a better interacting partner than BX-912, and BX-795. The present study will provide insights to know the particular residues which are involved in ligand binding. All these observations clearly indicate that OTSSP167 showed better specificity and binding efficiency with a MARK4 bearing stable interactions with key residues, thereby making them tough ATP competitors. The closure of the catalytic cleft observed in the ligand bound complexes and its independency to the movement of the T-loop makes them promising candidates in hampering the role of MARK4 in various diseases. Thus, its derivatives can be used as therapeutic target against various life threatening diseases.