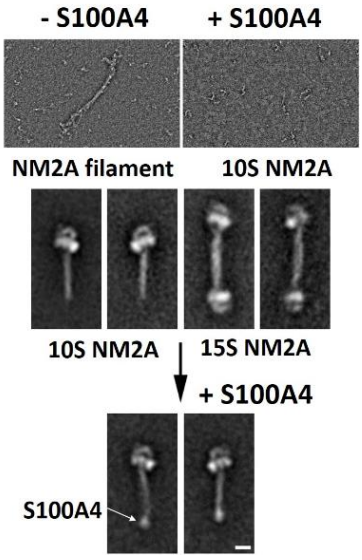
FINAL REPORT OF K108437 GRANT

Structural and functional studies of the Ca^{2+}-binding S100A4 protein involved in metastasis and chronic inflammation

Isoform selectivity of S100A4 binding to non-muscle myosin 2 (NM2):

We searched for major determinants of this selectivity by studying the roles of functional NM2 segments. Based on paralog scanning using phage-display we identified a single position (1907, NM2A numbering) as major determinant of isoform selective S100A4 binding. The structural background of this can be explained in part by a communication between the consecutive α-helical binding segments. This communication is completely abolished by the Ala-to-Asn substitution. By the mutual swapping of the non-helical S100A4-binding regions of NM2 isoforms, we demonstrated that the non-helical tail only slightly affected the affinity of the NM2 chimeras. Finally, we also found that the higher stability of the C-terminal proximal coiled-coil region of NM2B also discriminates against binding of S100A4 to this isoform. Our results clearly show that the isoform-selective binding of S100A4 to NM2s is determined at multiple levels in the sequence and structure of the three NM2 isoforms and the corresponding functional elements of NM2 act synergistically with one another resulting in a complex interaction network. (**Kiss et al.: FEBS J., 2016**)



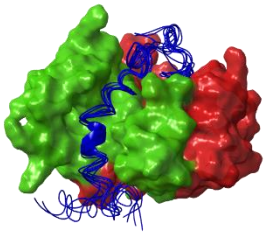
Further studies were conducted using full-length NM2 isoforms in collaboration Jim Sellers (NIH). We have established that in the presence of S100A4 NM2A minifilaments are disrupted and only monomeric so-call 10 S folded NM2A molecules are visible by electron microscopy (with visible S100A4 at the C-terminal tail tips (Fig. 1). These experiments are continued as part of our new grant proposal with the goal of comparing the regulatory roles of S100A4 and tailpiece phosphorylation (by casein kinase 2) in myosin minifilament dynamics.

Figure 1: *EM pictures showing that S100A4 disrupts NM2 filaments and can be visualized at the tip of folded 10S monomers (bar: 10 nm)*

Structural characterization of S100A4 binding to its interacting partners by NMR spectroscopy:

We extensively investigate the solution characteristics of S100A4 interaction with NM2 isoforms. We characterized the dynamic behavior of the Ca^{2+}-loaded human S100A4 protein and the S100A4-NM2A complex. It has been found that the 45-residue fragment is disordered in the free form and folds up into helical segments upon complex formation confirming the crystal structure observed previously. Importantly, some transient secondary structure formation was observed in the myosin fragment alone that could be important during the binding-folding mechanism. The asymmetric complex of Ca^{2+}-loaded S100A4 with has high stability and highly increased Ca^{2+}-affinity. We investigated the possible causes of

this allosteric effect by NMR spectroscopic approaches. Chemical shift based secondary structure analysis did not show substantial changes for the complex. Backbone dynamics revealed slow time-scale local motions in the H1 helices, which diminished in the complex and could be accompanied by an increase in dimer stability. Different mobilities in the Ca^{2+}-coordinating EF-hand sites indicate that they communicate by an allosteric mechanism operating through changes in protein dynamics that must be responsible for the elevated Ca^{2+}-affinity. **(Pálfy et al, Chembiochem, 2016)**



We also made progress in getting the atomic-resolution asymmetric structure of S100A4 with the TAD domain of p53 using NMR spectroscopy, SAXS and MD simulations (Fig. 2), however we still need some confirming studies before publication.

Figure 2. Molecular dynamic simulation of S100A4-p53 TAD domain based on some NMR constrains and SAXS data.

Characterization of the interaction of other S100 isoforms with NM2 isoforms:

Binding assays of S100P and S100A2 with NM2A, NM2B, NM2C were performed by FP and ITC methods. We could not reproduce literature data of high affinity binding of S100P to NM2A, however obtained submicromolar affinity between S100A2 (the closest relative of S100A4 in the S100 family) and NM2A and NM2C. We failed to get crystals of S100A2 and NM2A (similar to S100A4 and NM2C). No binding of S100B to either NM isoforms was found. These results were used in an MSc thesis. (Andrea Gáspári: Characterization of the protein-protein interactions of S100 paralogs with non-muscle myosin 2 isoforms; Eötvös Loránd University, 2014)

Role of disulfide formation in S100A4 function:

We made considerable effort to characterize oxidation of S100A4 since this posttranslational modification likely affect the function of the extracellular form of this protein. We characterized how oxidation of wild-type and various variants of S100A4 affect its structure and its interaction with an NM2A peptide. Wild-type S100A4 protein under reducing conditions showed no evidence of oligomerization by gel filtration chromatography and analysis of the SAXS data, thus I disproved previously reports. Oxidation the wild-type S100A4 protein displayed considerable heterogeneity, suggesting the several different crosslinked species are formed. Small amount of trimers and tetramers appeared too, however most of the disulfide-bonded S100A4 were found to be dimers. We created four S100A4 variants containing only a single cysteine to find out which cysteine is able to form disulfide bridges. Oxidation S100A4C3 variant did not confirm the hypothesis that oligomerization occurs through Cys3, since analysis of the SAXS data showed no signs of oligomers. CD spectroscopy studies showed decrease in α-helix, that is some structural changes in oxidize S100A4. FP measurements showed that after oxidation in the case of S100A4C81 the strong affinity to NM2A peptide decreased. As a result of oxidation of the S100A4C3 and the S100A4C76, we did not observed significant reduction in the affinity to NM2A peptide, suggesting that oxidation does not affect the structure of the binding

surface. In contrast, there was no measurable interaction of oxidized S100A4C86 with the NM2A peptides. Based on these results we hypothesize that extracellularly oxidized S100A4 has a slightly modified structure and binding surface and able to interact with other proteins, like cell surface receptors. These result were so far used in an MSc thesis and will be published later. (Zoltán Ligeti: Studies on the oxidation of the Ca^{2+}-binding S100A4 protein; Eötvös Loránd University, 2016)

Characterization of S100A4 interaction with transglutaminase 2 (TG2):

TG2, as a Ca^{2+}-dependent cross-linking enzyme has recently been suggested to mediate S100A4-dependent tumor cell migration. We provided evidence that S100A4 is an interacting partner and also specific amine donor of TG2. TG2 incorporates a glutamine donor peptide to Lys100 in the C-terminal random coil region of S100A4. Importantly, the enzyme activity is not necessary for the interaction: S100A4 also binds to TG2 in the presence of a specific inhibitor that keeps the enzyme in an open conformation, or to an enzymatically inactive mutant. We also found that S100A4 considerably enhances TG2-mediated adhesion of A431 epithelial carcinoma cells to the extracellular matrix. This role is independent of enzyme activity and requires the open conformation of TG2. We propose that S100A4 stabilizes the open conformation of TG2, which binds to its cell surface receptor in this state and increases cell adhesion. **(Biri et al, Biochem. J., 2016)**

TG2 also function as an isopeptidase cleaving the previously formed crosslinks. The biological significance of this activity has not been revealed yet mainly because of the lack of protein based method for its characterization. We, in collaboration with László Fésüs's group (Debrecen University) have developed a novel kinetic method for measuring isopeptidase activity of human TG2 by monitoring decrease in the fluorescence polarisation of a protein substrate previously formed by crosslinking fluorescently labelled glutamine donor FLpepT26 to S100A4 at a the penultimate lysine residue (as established in our previous work). The developed method could be applied to test mutant enzymes and compounds which influence isopeptidase activity of TG2. **(Thangaraju K et al, Anal. Biochem., 2016)**

Interaction of S100A4 with ezrin:

Ezrin belongs to the ERM (ezrin, radixin, moesin) protein family that has a role in cell morphology changes by linking actin filaments to the apical membrane of epithelial cells. It is highly expressed in a variety of human cancers and promotes metastasis. Using tryptophan fluorescence and stopped-flow kinetics, we showed that S100A4 binds to the N-terminal ERM domain (N-ERMAD) of ezrin with a micromolar affinity. The binding involves the F2 lobe of the N-ERMAD and follows an induced fit kinetic mechanism. Interestingly, S100A4 binds also to the unstructured C-terminal actin binding domain (C-ERMAD) with similar affinity. Using NMR spectroscopy, we characterized the complex of S100A4 with the C-ERMAD and demonstrated that no ternary complex is simultaneously formed with the two ezrin domains. Furthermore, we also showed that S100A4 co-localizes with ezrin in HEK-293T cells. However, S100A4 is unable to bind to and activate full-length ezrin in vitro indicating that the interaction of S100A4 with ezrin requires other regulatory events such as protein phosphorylation and/or membrane binding, shifting the conformational equilibrium of ezrin

towards the open state. As both proteins play an important role in promoting metastasis, the characterization of their interaction could shed more light on the molecular events contributing to this pathological process. **(Biri et al PLoSOne, under review)**

Annexin A2 interactions with S100 proteins and their regulation by phosphorylation:

Annexin A2 (ANXA2) has a versatile role in membrane-associated functions including membrane repair, lipid aggregation, endo- and exocytosis and is known to be regulated by post-translational modifications and protein-protein interactions occurring in the unstructured N-terminal domain (NTD). Our sequence and structure analysis revealed a short sequence motif which is responsible for clamping the NTD to the core domain (CTD). 3D structure determination by X-ray crystallography (2.9 and 3.4 Å structure of $ANXA2^{pY24}$, as well as 1.9 Å resolution structure of $ANXA2^{S26E}$), limited proteolysis and thermofluor experiments as well as MD simulations indicated that the flexibility of the NTD and CTD are interrelated and oppositely regulated by Tyr24 and Ser26 phosphorylation to decrease or increase, respectively, the conformational fluctuation of both domains. The 3D structure of ANXA2–S100A4 complex indicated that the asymmetric binding of S100A4 to ANXA2 causes the dislocation of the full NTD from the CTD and similarly to Ser26Glu mutation unmasks the concave side of ANXA2. In contrast, pTyr24 anchors the NTD to the CTD and hampers the membrane-bridging function. We found that this inhibition of ANXA2 function can be restored by S100A4 and S100A10 binding. Based on our results we provide a structural model for the regulation of ANXA2-mediated membrane aggregation by NTD phosphorylation and S100-binding. **(Ecsédi et al., Structure, 2017 under revision)**

Effect of extra- and intracellular S100A4 on cell adhesion, migration and collective behavior of epithelial carcinoma cells:

Effect of extracellular S100A4 on cell adhesion and migration of epithelial carcinoma cells: The aim of this subproject is to study the function of extracellular S100A4 and its mode of internalization. Adhesion of A431 epithelial carcinoma cell line was studied by real-time, label-free impedance-based assays. Cell migration was followed by a similar impedance-based transwell assay system where S100A4 was used as a chemotactic agent. According to our results, extracellular S100A4 reduced cell adhesion on fibronectin-coated surface in a concentration-dependent manner. S100A4 induces chemotaxis of cells at a 1 µM concentration optimum. Decrease of cell adhesion could be inhibited by anti-S100A4 antibodies. Analysis of mutants that do not bind to NM2A and other interacting partners demonstrated that an intact binding interface is required for the effect of S100A4 on cell adhesion. Interestingly, an oxidized oligomeric S100A4 also failed to reduce cell adhesion (structural characterization of the oxidized S100A4 is still in progress). Immunofluorescence studies revealed that S100A4 is internalized shortly after adding it to the medium and accumulates in

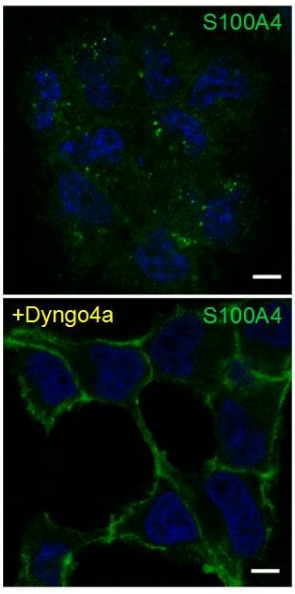


Fig. 3: Extracellularly added S100A4 internalized into early endosomes of A431 cells by a dynamin-dependent mechanism (nuclei: blue, anti-S100A4Ab: green)

endosomes (Fig. 3). The non-functional S100A4 mutants do not enter the cells, indicating that a specific receptor is involved in the process. (Biri et al, manuscript in preparation)

NM2-induced multicellular contractility is essential for development, maintenance and remodeling of tissue morphologies. To study NM2-dependent multicellular contractility, we developed two in vitro assays. The Matrigel contraction assay is sensitive to traction forces mediated through cell-ECM contacts, and it is interpreted by an elasto-plastic computational model. In this assay contractile forces are substantially reduced by low concentrations of NM2 inhibitor blebbistatin, Rho kinase inhibitor Y27632, extracellular S100A4 as well as overexpression of S100A4. The spheroid aggregation assay probes the contractility of the cortical cytoskeleton. NM2 inhibitors, at high concentrations in the medium, or overexpressed S100 reduce spheroid compactness. Interestingly, pharmacological NM2 inhibitors but not S100A4 initially elicit a transient, increased contraction of spheroids, but eventually they become less compact than spheroids in the absence of inhibitors. Thus, S100A4 regulates cell contractility in a distinct pattern than small molecule NM2 inhibitors or signaling through Rho kinase. Reduced multicellular contractility, in turn, yields looser aggregates thus enabling tumor cells to detach and metastasize. **(Biri et al, Mol Biol Cell, 2017, under revision)**

S100B interaction with RSK1 – a novel crosstalk of the Ras-MAPK and Ca-signaling pathways:

Overexpression of S100B in malignant melanomas significantly modulate MAPK signaling through a direct interaction with RSK1. Our biochemical X-ray crystallographic, solution small angle X-ray scattering and binding kinetics studies reveal the structural basis of this novel crosstalk interaction which could facilitate therapeutic targeting of melanomas. We have showed that S100B binds to a C-terminal RSK1 segment that is required not only for the MAPK (ERK2) recruitment but also for the autoinhibition of the RSK1 CaMK domain. Interestingly, S100B does not only directly interfere with the assembly of the ERK2-RSK1 heterodimeric complex, but it also negatively affects the activity of the CaMK domain of RSK1. Structural analysis revealed a highly dynamic, fuzzy complex. We also found that a single RSK1 fragment binds to an S100B dimer, representing the third asymmetric S100 complex determined by our group. Kinetic studies indicated that S100B binding to the CaMK domain involves both a conformational selection and an induced fit step. Based on the results of our structural analysis it was possible to assign a structural state to all observed kinetic steps. **(Gógl et al, J. Biol. Chem., 2016)**