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Abbreviations: AKT – protein kinase B; CacyBP/SIP – calyculin (S100A6) binding protein/Siah-1 interacting protein; CKII – casein kinase II; CS – domain present in CHORD-containing proteins and in Sgt1; EGFR – epidermal growth factor receptor; ERK1/2 – extracellular signal-regulated kinases; FAK – focal adhesion kinase; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; GSK3 β – glycogen synthase kinase 3 beta; Hsp – heat shock protein; IGF1R – insulin-like growth factor-binding protein 1; JNK – c-Jun N-terminal kinases; MAP – mitogen activated protein; MDM2 – mouse double minute 2; mTOR – mammalian ligand of rapamycin kinase; NFAT – nuclear factor of activated T cells; PAK – p21 activated kinase; PI3K – phosphoinositide 3-kinase; PKC – protein kinase C; PRELP – proline and arginine rich end leucine rich repeat protein; RAGE – receptor for advanced glycation end products; ROS – reactive oxygen species; SCF – Skp1/Cullin/F-box; SGS – Sgt1 specific; Sgt1 – suppressor of G2 allele of Skp1; STAT1 – signal transducer and activator of transcription 1; SUMO – small ubiquitin-related modifier; TPR – tetratricopeptide repeat

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ABSTRACT

S100A6, a calcium binding protein, whose gene was first identified as growth inducible one, has been linked to the process of cell proliferation and growth related phenomena ever since. While the structure and Ca²⁺ binding kinetics of S100A6 are rather well established the mechanism of its action has only recently begun to be elucidated. It is nonetheless evident that S100A6 exerts its biological role by interacting with a wide range of proteins ligands, many of which have been identified in our laboratory. Our research concentrates on two S100A6 ligands, CacyBP/SIP and Sgt1, which in turn possess their own interactomes. The imposing list of S100A6-interacting proteins indicates that together with its ligands it is a component of an extended network of cellular interactions and may be involved not only in cell proliferation but also in many other processes, of which cell differentiation and response to stress seem to be best documented.

INTRODUCTION

S100A6 is a Ca²⁺-binding protein discovered and characterized in our laboratory [Kuznicki & Filipek, 1987]. Over the years, due chiefly to the work performed in our laboratory, it has become evident that this protein can interact with many ligands and be part of different protein complexes. Since S100A6 interacts with other proteins only in a Ca²⁺-bound state, which it adopts following an increase in Ca²⁺ concentration, it confers Ca²⁺ sensitivity to all its subsequent cellular interactions. The best characterized ligands of S100A6 include the CacyBP/SIP protein, also discovered in our laboratory [Filipek & Wojda, 1996; Filipek & Kuznicki, 1998], and the Sgt1 protein, discovered by Kitagawa *et al.* [1999] but found to interact with S100A6 by our group [Nowotny *et al.*, 2003]. The research concerning the functional role of S100A6 and its two ligands, CacyBP/SIP and Sgt1, demonstrates that all three proteins, although structurally different, are involved in cell proliferation, differentiation and response to stress. Thus, in this manuscript, we focus on recent data, obtained mainly in our laboratory, concerning the role of S100A6 and its two ligands, CacyBP/SIP and Sgt1, in these processes, and also bring to notice several other interesting aspects of their biology.

PROPERTIES OF THE S100A6 PROTEIN

S100A6 is a low molecular weight, Ca²⁺-binding, homodimeric protein originally isolated from Ehrlich ascites tumor cells [Kuznicki & Filipek, 1987]. It binds two Ca²⁺ per monomer through so-called EF-hand structures, each consisting of two α helices linked by a short loop region (Fig. 1). Conformational change elicited by Ca²⁺ binding exposes the hitherto hidden hydrophobic regions that serve as a platform for interaction with protein ligands (Tab. 1).

S100A6 is present in different mammalian cells and tissues with particularly high expression in fibroblasts and epithelial cells [Kuznicki *et al.*, 1992]. The protein is localized mainly in the cytoplasm but it may associate with the plasma membrane and nuclear envelope upon increase in intracellular Ca²⁺ concentration [Leśniak & Filipek, 1996; Stradal & Gimona, 1999]. Nuclear localization of S100A6 has also been documented [Vimalachandran *et al.*, 2005].

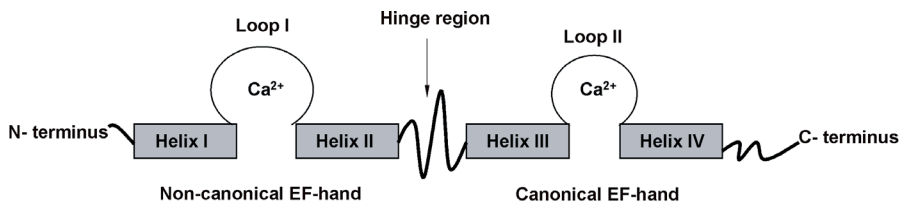


Figure 1. A schematic structure of S100A6 monomer.

Table 1. S100A6 ligands.

Cytoplasmic	Nuclear	Membrane	Extracellular
Annexins: II, VI, XI; tropomyosin, tubulin, caldesmon, calponin, CacyBP/SIP, Sgt1, melusin, Hop, FKBP38, FKBP52, CHIP, Cyp40, PP5, kinesin light chain, ERC-55, GAPDH, lysozyme	Lamin A/C, p53, p63, p73, importin α , FOR20, FOP, OFD	RAGE, integrin β	Lumican, PRELP, IGFBP-1

S100A6 is involved in many cellular processes but its primary function seems to be associated with cell proliferation and, consequently, cell differentiation. Another interesting aspect of S100A6 biology and function that has emerged recently is its involvement in cellular stress response due to interaction with chaperone and co-chaperone proteins.

S100A6 LIGANDS

S100A6 binds numerous protein ligands. The earliest identified were GAPDH (glyceraldehyde-3-phosphate dehydrogenase), annexins II, VI and XI, tropomyosin, caldesmon, calponin and lysozyme [reviewed in Leśniak *et al.*, 2009]. The cytoskeletal ligands of S100A6 have been recently complemented by α and β tubulin [Jurewicz *et al.*, 2018]. Binding to cytoskeletal proteins suggests that S100A6 could play a regulatory role in cytoskeleton organization [Filipek, 2001] while interaction with annexins implicates it in membrane dynamics. In fact, we have shown that in the presence of Ca^{2+} , S100A6 readily associates with membranes [Leśniak & Filipek, 1996]. Another set of S100A6 ligands includes transcription factors of the p53 family, namely p53, p63 and p73 [Słomnicki *et al.*, 2009; van Dieck *et al.*, 2010a], and ubiquitin ligase MDM2 (mouse double minute 2) [van Dieck *et al.*, 2010b]. This ligand set implicates S100A6 in regulation of the stability/activity of p53 family transcription factors. Indeed, S100A6 binding to p53 affects p53 tetramerization and interferes with p53 binding to MDM2 [van Dieck *et al.*, 2010b] and p300 acetyltransferase [Graczyk *et al.*, 2013]. We have also found that S100A6 can interact with lamin A/C [Kilańczyk *et al.*, 2012a], a nuclear protein involved in chromatin organization and implicated in colorectal cancer development and progression [Belt *et al.*, 2011; Willis *et al.*, 2008] while Takata *et al.* [2010] demonstrated the binding of S100A6 with the armadillo repeats of a nuclear transporter, importin α . These latter ligands implicate S100A6 in nuclear transport and the maintenance of nuclear structure but up to now there are no functional data concerning these interactions. Quite recently three centrosomal proteins, possibly involved in cilia formation, namely FOR20, FOP and OFD have been added to the list of S100A6 ligands [Sakane *et al.*, 2017]. Last but not least, research concerning S100A6 revealed a structurally similar but functionally diverse group of S100A6 ligands consisting of TPR or CS domain - containing proteins. This group includes Sgt1, melusin, Hop (Hsp90/Hsp70-organizing protein), kinesin light chain, Tom70 (translocase of outer mitochondrial membrane 70), FKBP52, Cyp40 (cyclophilin 40), CHIP (C-terminus of Hsc70-interacting protein), CacyBP/SIP, PP5 (protein phosphatase 5) and FKBP38 [Nowotny *et al.*, 2003; Filipek

et al. 2008a; Shimamoto *et al.*, 2010; Shimamoto *et al.*, 2014; Yamaguchi *et al.*, 2012].

S100A6 can be secreted from cells, probably by a microtubule - dependent mechanism [Jurewicz *et al.*, 2018] and can be detected in extracellular matrix and various body fluids including blood [reviewed in Leśniak *et al.*, 2017]. Extracellular ligands of S100A6 include RAGE (receptor for advanced glycation end products) [Leclerc *et al.*, 2007] and integrin β 1 [Jurewicz *et al.*, 2014a] as well as non-receptor proteins and glycoproteins such as lumican, PRELP and IGFBP-1 [Jurewicz *et al.*, 2014b]. Our laboratory contributed largely to the list of the currently known S100A6 ligands and can boast identification of S100A6 interaction with: GAPDH, lysozyme, calponin, p53, lamin A/C, CacyBP/SIP, Sgt1, melusin, integrin β 1, lumican, PRELP and IGFBP-1.

INVOLVEMENT OF S100A6 IN CELL PROLIFERATION AND DIFFERENTIATION

S100A6 has been implicated in the phenomenon of cell growth and proliferation since the discovery of its mRNA among those most elevated when quiescent cells were stimulated by serum addition [Calabretta *et al.*, 1986]. Since then there have been many reports documenting the link between high S100A6 level and cell proliferation and motility, often leading to tumor growth and invasiveness. On the other hand low S100A6 used to be associated with opposite effects [reviewed in Leśniak *et al.*, 2009]. Of note, we were the first to notice that S100A6 deficiency not only inhibited cell proliferation but also affected the cell cycle and, if sustained, induced senescence in NIH3T3 fibroblasts [Słomnicki *et al.*, 2010].

Recently, much research effort has been put into determining molecular mechanism(s) by which S100A6 exerts its pro-survival functions. At least three reports show that overexpression of S100A6 results in an increase in mRNA and protein levels of β -catenin [Chen *et al.*, 2015; Liu *et al.*, 2015] or in its translocation to the nucleus [Li *et al.*, 2014]. β -catenin is a key mediator of the canonical Wnt signaling pathway first described for its role in carcinogenesis by Rijsewijk *et al.* [1987]. Given that β -catenin enhances S100A6 expression [Kilańczyk *et al.*, 2012a], a positive feedback loop supporting cell proliferation might be at work. Other reports point to activation of the MAP kinase signaling pathway by S100A6. Namely, a study performed on colorectal cancer cell lines showed increased phosphorylation (activation) of p38 and ERK1/2 kinases upon S100A6 overexpression and a corresponding drop in phosphorylation following S100A6 knock-down [Duan *et al.*, 2014]. Increased p38 was also detected after S100A6 overexpression in nasopharyngeal carcinoma cells, while

an adverse effect was observed after S100A6 silencing [Li *et al.*, 2017]. Another report implicated S100A6 in activation of yet another signaling pathway, namely the PI3K/AKT pathway [Li *et al.*, 2018]. Thus, experimental data link S100A6 with several highly pro-survival signaling pathways although we still do not know at which point the signal from S100A6 is integrated. In this respect our recent studies revealed that S100A6 overexpression in HaCaT keratinocytes led to upregulation of the level and to enhanced phosphorylation/activity of EGFR (epidermal growth factor receptor) [Leśniak, manuscript in preparation]. Since, at least in epidermal cells, EGFR is the central hub transmitting signals that promote proliferation and oppose apoptosis (through activation of the MAP kinase and AKT-PI3K-mTOR pathways), the possible involvement of S100A6 in modulation of EGFR activity would explain many of its biological effects. It is also worth to mention that extracellular S100A6, acting through RAGE, activated JNK kinase [Leclerc *et al.*, 2007] while our group demonstrated that its interaction with integrin β 1 activated the following downstream kinases: pGSK3 β , pFAK and pPAK [Jurewicz *et al.*, 2018]. Interestingly, recent results show that S100A6 may not only promote pro-survival signaling but also attenuate anti-proliferative pathways. Microarray analysis, supported by experimental data, revealed that S100A6 activated PIAS1, an inhibitor of STAT1, and thereby prevented expression of anti-proliferative genes [Lerchenmuller *et al.*, 2016].

Since cell proliferation and differentiation are tightly intertwined it seems obvious that S100A6 also has a say in the latter process but direct experimental data concerning the impact of S100A6 on cell differentiation are scarce. We have investigated this issue in detail using the model of epidermal differentiation [Graczyk & Leśniak, 2014]. In this model the stage of keratinocyte differentiation can be easily evaluated based on expression of specific epidermal proteins. As could be expected, S100A6 overexpression delayed the appearance of epidermal differentiation markers both in classic and organotypic keratinocyte cultures. On the other hand, S100A6 deficiency had no or only a slight effect on the pace of differentiation [Graczyk & Leśniak, 2014]. This suggests that S100A6, by propelling the opposite process, i.e., cell proliferation, impedes cell differentiation but does not actively inhibit it since S100A6 knock-down was not sufficient to foster differentiation. The results obtained by Li *et al.* [2015] on the osteogenic differentiation model could be interpreted essentially in the same way. Namely, it was demonstrated that S100A6 knock-down induced ALP (alkaline phosphatase; a marker of osteogenic differentiation) activity in mouse embryonic fibroblasts but not to a degree sufficient to induce ectopic bone formation. However, when differentiation was stimulated by addition of BMP9, one of the most potent osteogenic factors, cells with S100A6 knock-down produced significantly more bone mass compared to control cells. Conversely, S100A6 overexpression did not significantly affect BMP9-induced differentiation i.e., could not counteract a strong physiological differentiation signal.

Heat shock proteins, Hsp90 and Hsp70, are central primary chaperones that guard cellular homeostasis against assaults such as temperature increase, enhanced ROS (reactive oxygen species) generation and the like. These proteins cooperate in controlling proper folding, assembly and maturation of proteins (termed "client proteins"), both newly synthesized or misfolded due to stress conditions [Johnson *et al.*, 2012; Li *et al.*, 2012]. They do that in tight association with co-chaperone proteins. The chaperoning process is initiated by interaction between Hsp70 and the client protein, followed by transfer of the newly formed complex to the Hsp90-co-chaperone pair and formation of a quaternary complex (Hsp70-client protein-Hsp90-co-chaperone) called foldosome. Of note, studies performed in our laboratory, led to identification of two novel Hsp70/Hsp90-interacting co-chaperones, Sgt1 [Spiechowicz *et al.*, 2007] and CacyBP/SIP [Góral *et al.*, 2016].

There are numerous experimental data documenting the interaction of S100A6 with Hsp70/90-interacting co-chaperones. The list includes Hop (Hsp90/Hsp70-organizing protein), KLC (kinesin light chain), Tom70 (translocase of outer mitochondrial membrane 70), FKBP52, Cyp40 (cyclophilin 40), CHIP (C-terminus of Hsc70-interacting protein), PP5 (protein phosphatase 5), FKBP38 [Shimamoto *et al.*, 2008; Shimamoto *et al.*, 2010; Yamaguchi *et al.*, 2012], CacyBP/SIP [Filipek & Wojda 1996], Sgt1 [Nowotny *et al.*, 2003] and melusin [Filipek *et al.*, 2008a; Sbroggio *et al.*, 2008]. Furthermore, structural studies have demonstrated that interaction with S100A6 engages the same domain, namely the TPR domain, or the CS and/or SGS domain in the case of melusin and Sgt1, through which the co-chaperones interact with Hsp90 [Shimamoto *et al.*, 2008; Shimamoto *et al.*, 2010; Yamaguchi *et al.*, 2012; Lee *et al.*, 2004]. Accordingly, it was shown, both using recombinant proteins and cell lysates, that S100A6 competes with Hsp70/Hsp90 for binding with co-chaperone proteins e.g., HOP and KLC [Shimamoto *et al.*, 2008], FKBP38 [Shimamoto *et al.*, 2014] and Sgt1 [Spiechowicz *et al.*, 2007]. In the case of FKBP38, whose unique feature is that it interacts with Bcl-2 and BclX1, two anti-apoptotic proteins, it was demonstrated that S100A6 interferes with the FKBP38-Bcl-2 interaction as well [Shimamoto *et al.*, 2014]. Thus, there is a plethora of biochemical and structural evidence that S100A6 can attenuate or even disrupt the interaction between chaperones and co-chaperones, but the biological consequences of such action are still obscure. However, taking into account that S100A6 can bind to the above mentioned co-chaperones and modulate their interaction with Hsp70/Hsp90 only if Ca²⁺-loaded, an additional regulatory level of the chaperoning process, dependent on calcium signaling, becomes evident. In the context of the possible role of S100A6 in this process it should be mentioned that cellular S100A6 level increases in response to such stressful conditions as ischemia [Lewington *et al.*, 1997], hypertension [Kasacka *et al.*, 2016], mechanical stress [Breen *et al.*, 1999], irradiation [Orre *et al.*, 2007] and oxidative stress [Leśniak *et al.*, 2005].

Recently, the level and distribution of S100A6 in various brain structures of mice subjected to mild chronic stress have been examined in our laboratory [Bartkowska *et al.*, 2017]. A marked change in protein level of S100A6 was observed in brainstem structures of stressed mice in comparison to the control group. A decreased level of S100A6 in stressed animals was also detected in the olfactory bulb, cerebellum and stress-related structures such as the hippocampus and the hypothalamus. These observations suggest that S100A6 expression in neurons of mouse brain is affected by stressful conditions to which the animals are exposed.

DISCOVERY AND PROPERTIES OF CacyBP/SIP

The CacyBP/SIP protein was originally discovered in mouse Ehrlich ascites tumor cells as an S100A6 (calcyclin) ligand [Filipek & Wojda, 1996; Filipek & Kuźnicki, 1998] and later found to be a Siah-1 binding partner [Matsuzawa & Reed, 2001]. CacyBP/SIP is expressed in different mammalian cells and tissues. A high level of this protein can be detected in brain and spleen. In brain CacyBP/SIP is present mainly in neurons and the level of its mRNA changes during development [Jastrzebska *et al.*, 2000]. Studies on neuroblastoma cell lines, NB2a and SH-SY5Y, have shown that CacyBP/SIP is mainly a cytosolic protein but it may also translocate to the perinuclear region or to the nucleus upon increase in intracellular Ca^{2+} concentration, retinoic acid treatment and oxidative stress [Filipek *et al.*, 2002a; Wu *et al.*, 2003; Topolska-Woś *et al.*, 2015]. Regarding the latter localization, our recent results have shown that CacyBP/SIP interacts with a nucleolar protein, NPM1, and has an impact on the amount of RNA bound to NPM1 [Rosinska & Filipek 2018]. Also, it has been found that CacyBP/SIP may serve as a regulator of transcriptional responses [Kilańczyk *et al.*, 2015, Rosińska *et al.*, 2016].

CacyBP/SIP consists of three structurally independent domains, the N-terminal, central – CS and the C-terminal – SGS [Bhattacharya *et al.*, 2005]. Interestingly, CacyBP/SIP was found to form dimers *in vitro* and in the cell and the N-terminal globular domain of CacyBP/SIP was defined as the one responsible for dimerization [Santelli *et al.*, 2005; Topolska-Woś *et al.*, 2015].

CacyBP/SIP LIGANDS

CacyBP/SIP is a multi-ligand protein. It binds S100 proteins, components of E3 ubiquitin ligases, Siah-1 and Skp1, cytoskeletal proteins such as tubulin, actin, tropomyosin and tau, MAP kinases such as ERK1/2 and p38, heat shock protein Hsp90 and nuclear proteins such as NPM1. Majority of CacyBP/SIP ligands were identified in our laboratory.

Siah-1 binds to the PAAVVAP motif present in the N-terminal domain of CacyBP/SIP while Skp1 - to its CS domain [Matsuzawa & Reed, 2001; Filipek *et al.*, 2002b; Bhattacharya *et al.*, 2005]. The binding of Skp1 to CacyBP/SIP seems to be transient since NMR (nuclear magnetic reso-

nance) studies did not reveal any significant structural perturbations of the CS domain upon binding of Skp1. A function for CacyBP/SIP-Siah-1-Skp1 interactions within the putative SCF^{TBL1} E3 ubiquitin ligase may involve p53-induced degradation of the non-phosphorylated form of an oncogene, namely, β -catenin [Matsuzawa & Reed, 2001; Liu *et al.*, 2001; Dimitrova *et al.*, 2010]. It has also been suggested that the major role of CacyBP/SIP in this ligase is to stabilize the association of its various components and orchestrate the correct distance and orientation of the ligase towards β -catenin.

Tubulin, actin, tropomyosin and tau are cytoskeletal proteins interacting with CacyBP/SIP that were identified by us [Filipek & Góral, 2018]. These interactions seem to be mainly responsible for cell proliferation, differentiation and, in consequence, for development, aging and neurodegeneration [Au *et al.*, 2006; Schneider *et al.*, 2007; Filipek *et al.*, 2008b; Schneider *et al.*, 2010; Jurewicz *et al.*, 2013; Wasik *et al.*, 2013; Czeredys *et al.*, 2013]. It has been suggested that interaction of CacyBP/SIP with tubulin might be responsible for tubulin oligomer transport during neurite outgrowth. Since some proteins are known to add long oligomers of tubulin to the growing end of a microtubule, CacyBP/SIP, through formation of tubulin assemblies, might speed up this process. Interaction of CacyBP/SIP with actin and tropomyosin might be important in microfilament organization in the growth cones. Regarding aging and neurodegeneration it was found that in neurons of 1) rat brain during physiological aging, 2) Alzheimer's disease (AD) brain and 3) tauopathic mouse brain (a model of AD) [Filipek *et al.*, 2008b; Wasik *et al.*, 2013], CacyBP/SIP is present mainly in neuronal somata and co-localizes with β -tubulin and tau. Thus, interaction of CacyBP/SIP with cytoskeletal proteins, at least in brain cells, seems to be involved in physiological development, aging and neurodegeneration.

Quite recently we have found that CacyBP/SIP binds and dephosphorylates the extracellular signal-regulated kinases ERK1/2 [Kilańczyk *et al.*, 2009; 2011]. Amino acid sequence analysis revealed important similarities with sequences of phosphatase-like proteins and certain MAP kinase phosphatases. The phosphatase activity of CacyBP/SIP towards ERK1/2 was demonstrated *in vitro* and also in neuroblastoma NB2a cells [Kilańczyk *et al.*, 2012b; Rosińska *et al.*, 2016]. In particular, overexpression of CacyBP/SIP correlated with a reduction in the amount of phosphorylated ERK1/2 in the nuclear fraction. In addition to ERK1/2, also p38 and tau were found to be potential ligands of the CacyBP/SIP phosphatase, both *in vitro* and in NB2a cells. Interestingly, dephosphorylation of p38 by CacyBP/SIP in NB2a cells treated with hydrogen peroxide (H_2O_2) was much more effective than in untreated ones [Topolska-Woś *et al.*, 2017]. In conclusion, involvement of CacyBP/SIP in regulation of MAP kinases, ERK1/2 and p38, might point to the function of CacyBP/SIP in pro-survival but also in pro-apoptotic pathways.

S100A6 was the first identified binding partner of CacyBP/SIP [Filipek & Wojda, 1996]. The binding site for S100A6, and for other S100 proteins, is located within the

C-terminal, SGS, domain of CacyBP/SIP [Nowotny *et al.*, 2000; Lee *et al.*, 2008]. S100A6 binding depends on Ca^{2+} concentration and occurs at its physiological range [Filipek & Kuźnicki, 1998]. The function of S100A6-CacyBP/SIP complexes is not entirely clear but multiple evidence suggests that S100A6 may modify CacyBP/SIP interactions with other proteins and thereby affect its function. For example, it has been suggested that, *via* binding to CacyBP/SIP, S100A6 may inhibit the activity of the putative SCF^{TBL1} E3 ubiquitin ligase [Ning *et al.*, 2012]. In consequence this might lead to higher β -catenin level and cancer cell proliferation. Also, it was found that S100A6 regulates the CacyBP/SIP-ERK1/2 interaction [Kilańczyk *et al.* 2009] and inhibits phosphatase activity of CacyBP/SIP toward tau [Wasik *et al.*, 2013]. This observation is consistent with a Ca^{2+} -dependent regulatory mechanism of protein dephosphorylation described previously for other S100 family members [Yamaguchi, *et al.*, 2012].

It is worthy to mention that phosphatase activity of CacyBP/SIP depends on its posttranslational modifications, mainly on phosphorylation. Analysis of mouse CacyBP/SIP phosphorylation in NB2a cells revealed that serine 22 and threonine 23 were phosphorylated by PKC and that this, in turn, increased the phosphatase activity towards ERK1/2 [Kilańczyk *et al.*, 2012b]. Moreover, it has been reported that CacyBP/SIP can be phosphorylated on threonine 184 by CKII (casein kinase II) [Wasik *et al.*, 2016]. In the presence of Ca^{2+} , phosphorylation of this residue is inhibited by S100A6, presumably due to competition between S100A6 and CKII kinase for binding to CacyBP/SIP. In addition to phosphorylation, CacyBP/SIP undergoes sumoylation, i.e. covalent attachment of a SUMO (Small Ubiquitin Related-Modifier) moiety to the ϵ -amino group of a lysine residue [Wasik & Filipek, 2013]. Binding of CacyBP/SIP to the SUMO E2-conjugating enzyme, Ubc9, was observed in NB2a cell lysates and the SUMO attachment site was identified as lysine 16. Interestingly, unlike the majority of sumoylated proteins, CacyBP/SIP with covalently bound SUMO was detected in the cytoplasmic and not in the nuclear fraction.

CacyBP/SIP IN CELL PROLIFERATION AND DIFFERENTIATION

The role of CacyBP/SIP in cell proliferation, mainly in development of different tumors, has been extensively studied. High expression of CacyBP/SIP was found in pancreatic cancer, gastric and colon cancer, nasopharyngeal carcinoma, osteogenic sarcoma, melanoma and breast cancer [Zhai *et al.*, 2008; Ning *et al.*, 2016]. Concerning the expression of CacyBP/SIP in breast cancer, the results obtained up to now are inconsistent [Wang *et al.*, 2010; Nie *et al.*, 2010; Kilańczyk *et al.*, 2014].

Proteomic analysis of HCT116 cells has indicated that overexpression of CacyBP/SIP inhibits cell proliferation although at the same time it promotes metastasis [Ghosh *et al.*, 2013]. On the contrary, our results demonstrate that overexpression of CacyBP/SIP slightly promotes proliferation of HCT116 cells while silencing inhibits this process [Kądziołka *et al.*, 2018]. A stimulatory effect of CacyBP/SIP overexpression on proliferation was also

observed for various other colon cancer cell lines [Zhai *et al.*, 2017]. Although reports mentioned above indicate the involvement of CacyBP/SIP in cell proliferation and tumorigenesis, the mechanism of its action in these processes is not well established. The one common issue is the perturbed level of β -catenin. As mentioned above, S100A6 may interfere with CacyBP/SIP functioning in the E3 ligase complex attenuate β -catenin degradation [Ning *et al.*, 2012]. These observations are consistent with the results showing that in some cancers, which are characterized by decreased CacyBP/SIP expression, β -catenin is up-regulated [Sun *et al.*, 2007].

Not only proliferation and tumorigenesis but also cell differentiation seems to be regulated by CacyBP/SIP in various cell types and tissues. The level of CacyBP/SIP was found to be increased in differentiating erythroid cells upon activation of erythropoietin receptor [Xia *et al.*, 2000; Pircher *et al.*, 2001], in thymocytes [Fukushima *et al.*, 2006], in endometrium of uterus during pregnancy of the mouse, and during the decidualization process i.e., development of the decidual tissue [Yang *et al.*, 2006; Herington *et al.*, 2007]. A possible role of CacyBP/SIP in differentiation of endometrial cells has been reported by Yang *et al.* [2006]. In this context, it is interesting that higher level of CacyBP/SIP was found in differentiated rat cardiomyocytes and neuroblastoma NB2a cells [Au *et al.*, 2006; Schneider *et al.*, 2007]. Involvement of CacyBP/SIP in cell differentiation might be also linked to dephosphorylation of ERK1/2. In differentiated NB2a cells in which CacyBP/SIP is up-regulated, the level of phosphorylated Elk-1, a transcription factor involved in cell proliferation, was reduced [Kilańczyk *et al.*, 2011], which in consequence might enhance differentiation. Overexpression of CacyBP/SIP in these cells led to activation of the pERK1/2-pCREB-BDNF pathway, associated with neuronal differentiation [Rosińska *et al.*, 2016].

CacyBP/SIP IN STRESS RESPONSE

Our recent results showed that CacyBP/SIP interacts with Hsp90 *in vitro* and in the cell and that inhibitors of Hsp90 do not perturb CacyBP/SIP-Hsp90 binding [Góral *et al.*, 2016]. Moreover, *in vitro* luciferase renaturation and citrate synthase aggregation assays have revealed that CacyBP/SIP itself exhibits chaperone properties. Our results also show that some stress factors, H_2O_2 or radicicol (Hsp90 inhibitor), resulted in a significant increase (up to 40-50%) in CacyBP/SIP level and that HEp-2 cells overexpressing CacyBP/SIP were more resistant to stress-induced death [Góral *et al.*, 2018]. Interestingly, our recent data obtained using NB2a cells indicate that CacyBP/SIP is indispensable for maintaining the NPM1 level and the structure of nucleoli under oxidative stress [Rosinska & Filipek 2018]. To check whether the CacyBP/SIP protein might play a role in stress responses *in vivo*, its level in selected brain structures of control and stressed mice was analyzed. It was found that CacyBP/SIP was more abundant in the thalamus/hypothalamus, hippocampus and brainstem of stressed mice [Góral *et al.*, 2018]. Thus, these results indicate that CacyBP/SIP is involved in formation of chaperone complexes and in cellular stress response.

It should be also mentioned that our recent data obtained from microarray analysis have shown that overexpression of CacyBP/SIP in HCT116 cells affects signaling pathways that regulate other processes including immune responses [Kądziołka *et al.*, 2018]. This is not surprising since earlier studies demonstrated a relative abundance of CacyBP/SIP in mouse spleen i.e., an organ of the lymphatic system [Filipek & Wojda 1996] and revealed that CacyBP/SIP knock-out mice had a smaller spleen and thymus [Fukushima *et al.*, 2006]. In accordance with these data is the observation that CacyBP/SIP gene expression can be regulated by NFAT1, a transcription factor involved in immune responses [Kądziołka *et al.*, 2017]. All these results suggest that there is an interplay between CacyBP/SIP and proteins/factors involved in the immune system known to be connected with tumorigenesis and stress responses.

DISCOVERY AND PROPERTIES OF Sgt1

The Sgt1 protein was originally discovered in yeast cells (*S. cerevisiae*) [Kitagawa *et al.*, 1999]. It was shown that in these cells Sgt1 binds to the Skp1 protein and through this interaction activates the CBF3 kinetochore complex and the SCF ubiquitin ligase complex [Kitagawa *et al.*, 1999; Steensgaard *et al.*, 2004]. Later, Sgt1 was detected in various plants and mammals and several of its ligand proteins were identified [Spiechowicz & Filipek, 2005]. Among them were intracellular NLR (Nod-like receptor) proteins such as Nod1, Nod2 and NALP3 [da Silva Correia *et al.*, 2007; Mayor *et al.*, 2007; Kadota *et al.*, 2010], chaperones including Hsp90, Hsp70 and Hsc70 [Lee *et al.*, 2004; Spiechowicz *et al.*, 2007; Noël *et al.*, 2007] and members of the S100 family [Nowotny *et al.*, 2003].

Sgt1 is composed of three major domains i.e., TPR, CS and SGS. These two latter domains are also present in CacyBP/SIP (see above). The TPR domain is present in several other proteins involved in cell cycle, transcription, protein transport and chaperoning activity [Blatch & Lassle, 1999]. The CS domain located in the middle of the molecule plays a role in protein-protein interaction and the third domain, SGS, located in the C-terminal part, was shown by our group to bind S100A6 and, most probably, other calcium binding proteins of the S100 family [Nowotny *et al.*, 2003]. Interestingly, in man, but not in mouse or rat, two isoforms of Sgt1 were identified, Sgt1A and Sgt1B, with molecular mass of about 38 kDa and 40 kDa, respectively [Niikura & Kitagawa 2003; Zou *et al.*, 2004]

Studies regarding the expression of Sgt1 in mammals revealed its high level in brain, liver, lung and testis. Results of Western blot analysis performed on brain tissue showed that two structures, cerebellum and cortex, contain the highest amount of Sgt1. Immunohistochemical analysis has shown that high amount of Sgt1 is present in Purkinje cells of the cerebellum and in glial cells of the white matter. Further analyses on human brain slices revealed the presence of Sgt1 in neurons of the cortex, hippocampus, cerebellum and in oligodendrocytes of the white matter [Spiechowicz *et al.*, 2006]. Quantita-

tive analysis of Sgt1-immunostained cells in the cortical regions of healthy aged versus Alzheimer disease (AD) brains showed lower density of stained cells in AD material. This suggests that Sgt1-immunopositive cells are selectively affected in certain cortical layers of AD brain. The presence of Sgt1 in post-mitotic neurons and the decrease in the density of these neurons in AD cerebral cortex point to Sgt1 as a possible marker of neurons degenerating in AD.

INVOLVEMENT OF Sgt1 IN CHAPERONE COMPLEXES AND CELL PROLIFERATION

The interaction of Sgt1 with Hsp90 and the potential role of the complex formed by these two proteins were described by several groups [Takahashi *et al.*, 2003; Bansal *et al.*, 2004; Lingelbach *et al.*, 2004; Rodrigo-Brenni *et al.*, 2004]. As mentioned above, we have shown that Sgt1 interacts with Hsps (Hsp90 and Hsp70) through the C-terminal domain, which also binds S100A6, and that S100A6 competes for the binding [Nowotny *et al.*, 2003; Spiechowicz *et al.*, 2007]

Our group has demonstrated that Sgt1 exhibits co-chaperone properties and may also act as a chaperone itself since it is able to protect proteins from thermal aggregation as shown by the citrate synthase assay [Zabka *et al.*, 2008]. Furthermore, we have shown for the first time that the level of Sgt1 increases due to stress conditions [Zabka *et al.*, 2008; Kapustian *et al.*, 2013] similarly to the level of Hsps. In the case of heat shock, the increase in Sgt1 level is caused by transcription stimulation of the Sgt1 gene by HSF-1 transcription factor that binds to the promoter. It is worth to mention that transcription of Hsp encoding genes is also regulated by HSF-1. These results may serve as an additional evidence that Sgt1 is a component of the chaperone complexes.

Interaction of Sgt1 with Hsp90 and possible involvement of this complex in cell proliferation prompted us to study the presence of Sgt1 in cell nucleus. Using different methods we have found that in HEp-2 cells Sgt1 translocated to the nucleus following heat shock or thapsigargin treatment. Interestingly, Sgt1 nuclear localization was observed at a much smaller scale in HEp-2 cells with diminished level of S100A6. Since thapsigargin was ineffective in cells with diminished level of S100A6 it suggests that increase in intracellular Ca²⁺ concentration, transduced by S100A6, is necessary for nuclear translocation/localization of Sgt1 [Prus & Filipek, 2011]. Interestingly, not only Ca²⁺/S100A6 but also phosphorylation of Sgt1 appears to be important for its nuclear translocation. To check the role of Sgt1 phosphorylation/dephosphorylation we constructed non-phosphorylatable Sgt1 mutants (S249A, S299A, S249/299A) or a phosphorylation mimic (S299D) mutant. It was found that the levels of S299A and S249/299A mutants in the nuclear fraction of HEp-2 cells after heat shock were higher than the level of wild type Sgt1. Moreover, it was shown that S100A6 is required for translocation of the non-phosphorylatable Sgt1 mutants and that upon heat shock S100A6 translocated to the nucleus together with Sgt1. In addition, it was found that

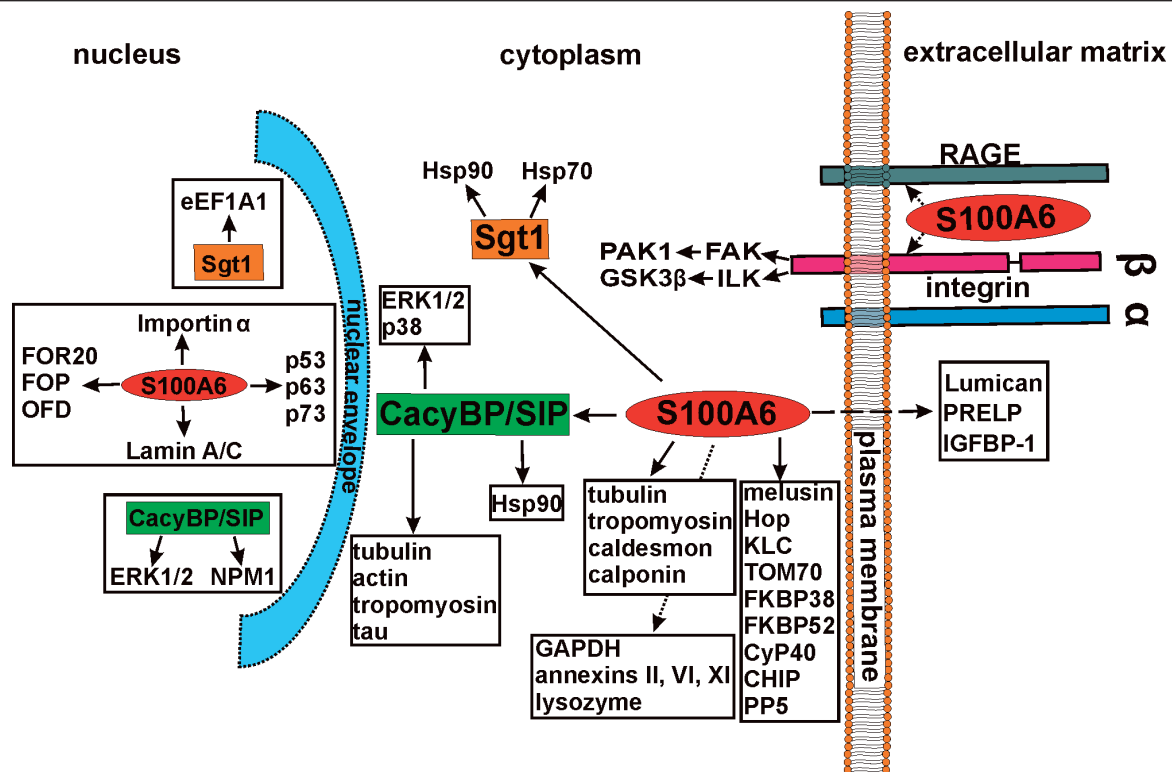


Figure 2. Selected interactions of S100A6 and its two ligands, CacyBP/SIP and Sgt1.

non-phosphorylatable Sgt1 mutant interacts with S100A6 more efficiently and at the same time exhibits lower affinity for Hsp90 than wild type Sgt1. Altogether, our results suggest that S100A6-Ca²⁺-mediated Sgt1 dephosphorylation promotes its nuclear translocation / localization, most probably due to disruption of the Sgt1-Hsp90 complex [Prus *et al.*, 2011].

Sgt1 AND PATHOGEN RESISTANCE

The majority of reports regarding the Sgt1 protein describe the properties and function of its plant homologue. For instance, it has been found that in plant, Sgt1 together with its ligand protein, RAR1, interacts with Hsp90 and modulates activity and stability of the essential components of signaling pathways leading to pathogen resistance [Shirasu & Schulze-Lefert, 2003; Takahashi *et al.*, 2003; Hubert *et al.*, 2003]. Plant Sgt1 was also found to bind to the ubiquitin-conjugating enzyme, Rad6, and to be involved in auxins' degradation *via* SCF^{TIR} ubiquitin ligase [Yamamoto *et al.*, 2004; Gray *et al.*, 2003]. Moreover, Sgt1 has been reported to play a role in host and non-host resistance [Peart *et al.*, 2002], which suggests that the protein might be a general factor in combating disease and restraining viral multiplication [Komatsu *et al.*, 2010].

To provide insights into the protein networks involved in antiviral defense in mammalian cells we have analyzed the interaction between human Sgt1 and the eEF1A protein. eEF1A is known to improve virus performance *via* interaction with viral genomic RNA and viral RNA-dependent RNA polymerase [Li *et al.*, 2013]. We have found that EF1A interacts with Sgt1 and, moreover, Sgt1 prevents eEF1A1 binding to viral RNA. The reversible character of

the competition between Sgt1 and viral RNA for eEF1A1 suggests a need for higher Sgt1 expression in the organism to combat the viral attack [Novosylna *et al.*, 2015].

CONCLUSIONS

Isolation and purification of a novel calcium binding protein, S100A6 (originally called calcylin), in 1987 started our journey along a fascinating albeit sometimes difficult path with occasional pitfalls but not without rewards. First considered as a "simple" Ca²⁺ buffering protein S100A6 soon proved to be an important element of the cell machinery, especially when cell proliferation, cancer invasion and metastasis were taken into account. With subsequent identification of S100A6 ligands, most of all CacyBP/SIP and Sgt1, the number of cellular processes in which these proteins appeared to be involved expanded rapidly. We can now say that the network of interactions formed by these proteins extends from the nucleus to the extracellular space and affects processes as diverse as maintenance of nuclear/nucleolar integrity, chaperoning activity, protein ubiquitination or receptor signaling (see Fig. 2). In this we have presented only a fragment of this network, concentrating on the impact of S100A6, CacyBP/SIP and Sgt1 on cell proliferation/differentiation and response to stress executed by cellular chaperones but, definitely, there is much more to tell.

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Aktualny pogląd na komórkową funkcję S100A6 i jego ligandów, CacyBP/SIP i Sgt1

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Słowa kluczowe: S100A6, CacyBP/SIP, Sgt1, Hsp90, proliferacja, różnicowanie, odpowiedź na stress

STRESZCZENIE

S100A6 jest białkiem wiążącym wapń, którego gen zidentyfikowano po raz pierwszy jako gen indukowany czynnikami wzrostu. Od tego czasu przyjmuje się, że S100A6 jest białkiem biorącym udział w proliferacji i pokrewnych procesach komórkowych. Podczas gdy struktura S100A6 i kinetyka wiązania Ca²⁺ zostały dość wcześnie określone to badania nad mechanizmem działania S100A6 są prowadzone stosunkowo od niedawna. Niemniej wydaje się oczywiste, że S100A6 pełni swoją funkcję w komórce poprzez oddziaływanie w wieloma ligandami, z których większość została zidentyfikowana w naszym zespole. Ostatnio nasze badania koncentrują się na dwóch ligandach S100A6, to jest białkach CacyBP/SIP i Sgt1, które posiadają też swoje własne białka docelowe. Długa lista ligandów S100A6, CacyBP/SIP i Sgt1 wskazuje, że białka te są składnikami złożonej sieci interakcji i mogą być zaangażowane nie tylko w proliferację ale również w wiele innych procesów komórkowych, z których różnicowanie czy odpowiedź na stres wydają się być najlepiej udokumentowane.