Heterocomplex formation between metastasis-related protein S100A4 (Mts1) and S100A1 as revealed by the yeast two-hybrid system

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Abstract S100A4 (Mts1) is a Ca^{2+}-binding protein of the S100 family. This protein plays an important role in promoting tumor metastasis. In order to identify S100A4 interacting proteins, we have applied the yeast two-hybrid system as an in vivo approach. By screening a mouse mammary adenocarcinoma library, we have demonstrated that S100A4 forms a heterocomplex with S100A1, another member of the S100 family. The non-covalent heterodimerization was confirmed by fluorescence spectroscopy and electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. Mutational analysis revealed that replacement of Cys^{76} and/or Cys^{81} of S100A4 by Ser abolishes the S100A4/S100A1 heterodimerization, but does not affect the S100A4 homodimerization in vivo.

Key words: S100 protein; Mts1; Two-hybrid system; Heterodimer

1. Introduction

Members of the S100 family have been implicated in a wide range of cellular processes including cell growth and differentiation, signal transduction, microtubule dynamics, cytoskeleton organization and cell motility [1,2]. The S100 proteins utilize two helix-loop-helix motifs, known as EF-hands, for calcium-binding [3]. The most puzzling feature of the S100 proteins is their link with different human diseases. Deregulated S100 expression is associated with breast cancer and melanoma, metastasis, Alzheimer's disease, cardiomyopathies, psoriasis and cystic fibrosis [2]. For this reason, the S100 proteins are widely used as molecular markers in pathology [4,5].

The biological effects mediated by the S100 proteins are determined by their interaction with cellular targets. Over the past few years, significant progress has been made towards identifying the target proteins that could relay regulatory signals of the S100 family members. This search revealed that cytoskeleton proteins [6,7], transcription factors [8], kinases [9] and annexins [10] are potential targets for S100.

The S100A4 protein is believed to be involved in triggering the cascade of events that lead to the metastatic phenotype of tumor cells. The level of S100A4 expression strongly correlates with the metastatic potential of different mouse tumors [11,12] and it was recently demonstrated by two different transgenic mouse models that overexpression of the S100A4 transgene causes an increase of the metastatic ability of breast tumors [13,14]. However, the molecular mechanism of the S100A4 action is not yet completely understood.

Here, we applied the yeast two-hybrid system as an in vivo approach to screen a mouse mammary adenocarcinoma cDNA library for S100A4 interacting proteins. The screening revealed the homodimerization of S100A4 as well as the interaction of S100A4 with another member of the S100 family, S100A1.

2. Materials and methods

2.1. GAL4-activating domain cDNA library construction

cDNA was synthesized on poly(A)$^+$ mRNA isolated from the CSML-100 mouse mammary adenocarcinoma cell line using oligo-$(dT)_{12}$ primer and cloned into the Hybri-ZAP phage vector (Hybri-ZAP two-hybrid vector kit, Stratagene). The library was amplified and converted into plasmids by the mass excision procedure according to the manufacturer's protocol.

2.2. The pBD-S100A4 bait plasmid

The open reading frame of the mouse *S100A4* gene (exons II–III) was cloned in-frame with the GAL4 DNA-binding domain into the yeast expression vector pBD-GAL4/Cam (Stratagene) as follows: the pBD-GAL4/Cam plasmid was digested with *Eco*RI, flushed with the Klenow fragment of DNA polymerase I and redigested with *Sal*I. The *S100A4* cDNA insert was generated by digesting the p271 plasmid [11] with *Nco*I, flushing with the Klenow polymerase and redigesting with *Sal*I. The *S100A4* cDNA was ligated with the pBD-GAL4/Cam vector resulting in the pBD-S100A4 bait plasmid. The construct was verified by dideoxy chain termination sequencing. Expression of the bait protein in yeast was confirmed by Western blotting with a monoclonal anti-S100A4 antibody.

2.3. Yeast transformation

The *Saccharomyces cerevisiae* strain YRG-2 (MATα *ura3-52 his3-299 ade2-101 lys2-801 trp1-901 leu2-3 112 gal4-542 gal80-538* LYS2::UAS_{GAL4}-$TATA_{GAL1}$-HIS3 URA3::UAS_{GAL4}-$TATA_{CYC1}$-*lacZ*) (Stratagene) was used for all assays. Yeast cultures were grown at 30°C in either YPD medium (2% peptone, 1% yeast extract, 2% glucose) or SD minimal medium (0.67% nitrogen base, 18.2% D-sor-

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Abbreviations: BD, DNA-binding domain of GAL4; AD, transcriptional activation domain of GAL4; PCR, polymerase chain reaction

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bitol, 2% glucose, supplemented with amino acids). Transformation of yeast was performed using the YRG-2 Yeast Competent Cell kit (Stratagene) according to the manufacturer's protocol. Yeasts transformed with pBD-S100A4 along with the GAL4-transactivating domain cDNA library were grown on SD agar plates lacking Trp, Leu and His. After 3–7 days, the HIS3 positive colonies were streaked on either a new SD agar plate without Trp, Leu, His or on a nitrocellulose membrane (Hybond C, Amersham Pharmacia Biotech) placed on a SD agar plate without Trp and Leu and grown for 1 or 3 days, respectively, at 30°C. The filter β-galactosidase assay was performed according to the protocol of Stratagene. The time required for color development ranged from 1 h to overnight.

2.4. Mutagenesis

All mutations were introduced into *S100A4* by polymerase chain reaction (PCR) using the pBD-S100A4 plasmid as a template with the following primers: $Cys^{76} \rightarrow Ser$ reverse: 5′-GACAGAGTACTCCTG-GAAGTC-3′; $Cys^{81} \rightarrow Ser$ forward: 5′-TTCCTGTCCTCCATTGC-CATG-3′; $Cys^{86} \rightarrow Ser$ reverse: 5′-GAATTCATTGGACATCATG-GC-3′; $Cys^{93} \rightarrow Ser$ forward: 5′-TTTGAGGGCTCCCCAGA\T-AAG-3′; $Phe^{72} \rightarrow Ala$ forward: 5′-GCCCAGGAGTACTGTG-TCTTCCT-3′; $Phe^{72} \rightarrow Ala$ reverse: 5′-GTCAACTTCATTGTC-CCTGT-3′; ΔLeu^{79} forward: 5′-TCCTGCATTGCCATGATGTG-CA-3′; ΔLeu^{79} reverse: 5′-GAAGACACAGTACTCCTGGA-3′.

PCR amplification was performed using a 10:1 AmpliTaq (Perkin Elmer):*Pfu*I (Stratagene) polymerase mixture. PCR products were eluted from an agarose gel, self-ligated and used for transformation of the XL-1 *Escherichia coli* strain. All constructs were verified by dideoxy chain termination sequencing [15].

2.5. Protein expression and purification

For overexpression of the recombinant S100 proteins in *E. coli* M15, the T5 RNA polymerase promoter-based expression vector pQE30 (Qiagen) was used. Expression of recombinant proteins was induced at an A_{600} value of 0.7 by 1 mM isopropyl-1-thio-β-D-galactopyranoside. The bacteria were harvested after 4 h by centrifugation. The pellet obtained from 2 l of bacterial culture was resuspended in 50 ml of 10 mM Tris–HCl pH 7.5, 0.1 M sucrose and frozen at −80°C. To purify the recombinant S100 proteins, the cell suspension was thawed and diluted 2-fold with 100 mM Tris–HCl, pH 7.5, 400 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol. The final suspension was sonicated with 8 bursts of 15 s at the maximum amplitude with 30 s intervals. The sonicated sample was centrifuged at $16\,000 \times g$ for 30 min. The resulting supernatant was brought to 5 mM of $CaCl_2$ and applied to a Phenyl-Sepharose column (Amersham Pharmacia Biotech) equilibrated with 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$. The recombinant proteins were eluted with 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, concentrated using Filtron 10K and their purification was completed by size exclusion chromatography. The purity of all proteins was determined by sodium dodecyl sulfate (SDS)–PAGE and mass spectrometry and their dimeric state was confirmed by analytical ultracentrifugation. Analytical centrifugation was performed as described in [16].

2.6. Fluorescence spectroscopy

Steady-state fluorescence experiments were carried out on a FluoroMax spectrofluorimeter equipped with a circulating water bath. Tryptophan specific excitation wavelength 295 nm was used. All readings were corrected for dilution and contribution of tyrosine emission of S100A4.

2.7. Electrospray ionization Fourier transform ion cyclotron resonance (ESI-FTICR) mass spectrometry

Mass spectrometry measurements were made using a FTICR mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with a shielded 9.4T super-conducting magnet (Magnex Scientific Ltd., Abingdon, UK), a cylindrical 'infinity' ICR cell with diameter $d = 0.06$ m and an external ESI source (Analytica of Branford, Branford, USA) as described previously [17]. The ESI source was equipped with a capillary made of pyrex, coated on both ends with nickel. Solutions of S100A1 and S100A4 at equimolar concentrations (typically 20 μM) were sprayed at room temperature from 5 mM ammonium acetate buffer (pH 6). Carbon dioxide was used as a drying gas in the electrospray source.

3. Results

The yeast two-hybrid system for screening cDNA encoding cellular proteins that are able to interact with a protein of interest has been applied in a large number of studies. It was successfully applied to study protein–protein interactions in complex biological processes such as cell proliferation, differentiation, apoptosis and intracellular signal transduction [18–20].

To identify proteins which could interact with S100A4, we co-transfected the YRG-2 yeast strain with the bait plasmid pBD-S100A4 and the CSML-100 mouse adenocarcinoma cDNA library. The interaction phenotype was scored by the ability of the transformed yeast to grow on a synthetic medium without histidine and by β-galactosidase activity. From 2×10^6 transformants, 287 colonies grew on a selective medium lacking histidine. Of these 287 colonies, 53 revealed β-galactosidase activity in a filter assay. Plasmid DNA from these colonies was isolated and subcloned in *E. coli*. All rescued plasmids were then re-introduced into the yeast along with pBD-S100A4 to verify interactions and to exclude false positives. Only 41 out of 53 originally isolated clones grew on

Table 1
Mutation analysis of the S100A4/S100A1 interaction using the yeast two-hybrid system

Bait (BD-GAL4 fusion)	Prey (AD-GAL4 fusion)	HIS3 phenotype	β-Gal phenotype
S100A4	S100A4	+	blue
	S100A1	+	blue
S100A4-$Phe^{72} \rightarrow Ala$	S100A4	−	white
	S100A1	−	white
S100A4-ΔLeu^{79}	S100A4	−	white
	S100A1	−	white
S100A4-$Cys^{76,81,86,93} \rightarrow Ser$	S100A4	+	blue
	S100A1	−	white
S100A4-$Cys^{86,93} \rightarrow Ser$	S100A4	+	blue
	S100A1	+	blue
S100A4-$Cys^{76,81} \rightarrow Ser$	S100A4	+	blue
	S100A1	−	white
S100A4-$Cys^{76} \rightarrow Ser$	S100A4	+	blue
	S100A1	−	white

Yeast clones co-transfected with the GAL4 DNA-binding domain (BD) and GAL4 transcriptional activation domain (AD) hybrid constructs were first grown on SD medium without Trp and Leu (selection for the plasmids) and then streaked onto a SD plate lacking histidine or on a nitrocellulose membrane. The HIS3 phenotype was scored after 3 days. β-Galactosidase activity was analyzed by a filter assay using 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside as a substrate.

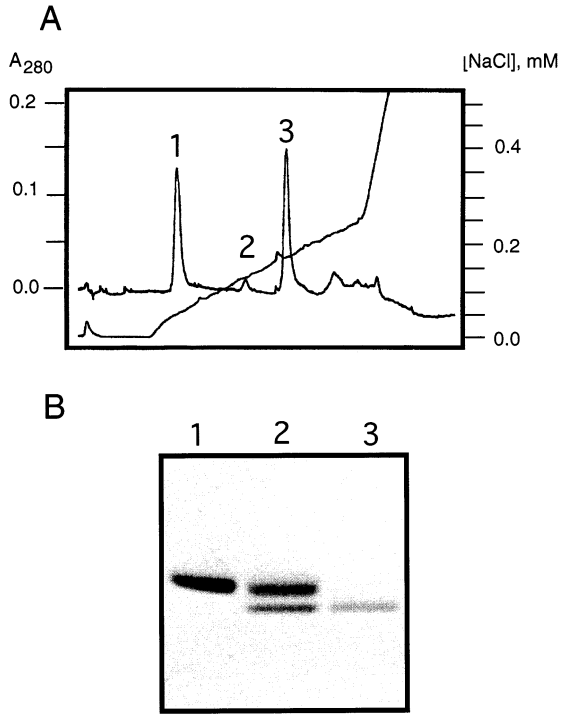

Fig. 1. Purification of the S100A1/A4 heterocomplex. A: S100A1 and S100A4 were obtained by overexpressing in *E. coli*. Proteins were purified as described in Section 2. To get heterocomplex, both proteins (S100A1/A4 molar ratio was 2:1) were incubated in 20 mM Tris–HCl, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.5 at room temperature for 30 min. The mixture was concentrated with a Filtron 10K microconcentrator to a final volume of 0.2 ml, diluted to 2 ml with 20 mM Tris–HCl, pH 7.5 (buffer A) and loaded on a 1 ml Resource Q10 column (Pharmacia) equilibrated with a same buffer. The elution was performed with a gradient of buffer B (buffer A+2 M NaCl). The flow rate was 2 ml/min and fractions of 2 ml were collected. B: The proteins were analyzed by 10% non-reduced Bis–Tris SDS–PAGE (Novex) in MES running buffer pH 7.3, followed by staining with Coomassie blue.

the selective medium without histidine. The corresponding cDNA was subjected to sequencing analysis.

Forty out of 41 clones were identified as the S100A4 cDNA fused in-frame with GAL4-AD, indicating the homodimerization of S100A4 in vivo. Besides the S100A4 dimers, one of the clones was identified as a cDNA encoding the S100A1 protein. To verify the interaction, the plasmids were transfected into yeast pairwise either with pBD-S100A4 and pLamin C or pBD-GAL4/Cam as controls. The S100A1 transfected yeast demonstrated S100A4 dependent transcription of the reporter genes, indicating the interaction (not shown).

To probe the region of S100A4 that is essential for heterodimerization, we tested several S100A4 mutant proteins for the interaction with S100A1 using the yeast two-hybrid system. The point mutations $Phe^{72} \rightarrow Ala$ and ΔLeu^{79}, which were shown to abolish the homodimerization of S100A4 (Tarabykina, S. et al., submitted), also affected the S100A4 interaction with S100A1 (Table 1). We have explored the cysteine scanning, since it was previously demonstrated for another S100 protein, S100B, that cysteines may play a role in S100 protein–protein interactions [21]. All four cysteines in S100A4 were replaced by serine in different combinations (Table 1). None of cysteines was essential for the S100A4 homodimerization. However, we have found that Cys^{76} and/or Cys^{81} play an important role in the S100A4/S100A1 heterodimerization. Two other cysteine residues Cys^{86} and Cys^{93} did not show any influence on the heterodimer formation in vivo.

An attempt was made to demonstrate homo- and heterodimerization of S100A4 and S100A1 by anion-exchange chromatography. Purified preparations of S100A4 and S100A1 were mixed and incubated without denaturation, then concentrated and fractionated on a Resource Q column. Several distinct peaks were detected (Fig. 1A). Two of them eluting at 50 mM NaCl and 180 mM NaCl were shown to be the S100A4 and S100A1 homodimers, respectively. The intermediate peak was very small and contained both S100A4 and S100A1 proteins. Only S100 monomers were observed on the SDS gel, which was run under non-reducing conditions, indicating the absence of covalently linked dimers (Fig. 1B). Although we had expected to see both protein species in the S100A4/S100A1 heterodimer in 1:1 ratio, the intensity of the S100A1 band was weaker than that of S100A4. To explain this observation, both proteins were applied onto an SDS gel in the same amounts (according to the Bradford assay and UV absorbance spectroscopy) and stained with Coomassie R250. We found that the staining intensities of the equal amounts of two recombinant proteins are different (not shown).

Direct evidence for the S100A4/S100A1 heterodimerization was obtained in vitro by ESI-FTICR mass spectrometry. Under appropriate conditions, the ESI technique allows the transportation of intact multisubunit complexes from their natural aqueous environment into the gas phase. We have previously applied ESI-FTICR mass spectrometry for the detection and characterization of the dimeric homocomplexes of both calmodulin and S100A4 ([22], Scott, D. et al., submitted). Here, we report the first direct observation of a heterocomplex formed between S100A1 and S100A4. Fig. 2 shows the ESI-FTICR mass spectrum obtained for an equimolar mixture of S100A1 and S100A4 in ammonium acetate buffer, pH 6. The ultra-high mass resolution obtained with the FTICR mass spectrometer allowed the isotopic resolution of each of the peaks observed and revealed that several different species were present. Signals corresponding to the species with the average masses of 11 864.16 and 13 117.45 Da, respectively, were observed and indicated the presence of both monomeric S100A1 and monomeric S100A4 proteins. However, the most abundant signal detected at *m/z* 2271 was found to correspond to the species carrying 11 charges with

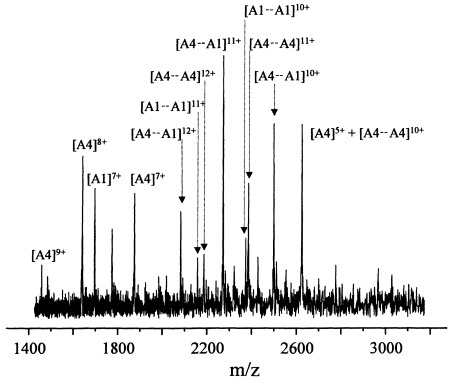

Fig. 2. ESI mass spectrum of S100A1 and S100A4 proteins sprayed from an aqueous 5 mM ammonium acetate buffer, pH 6.0.

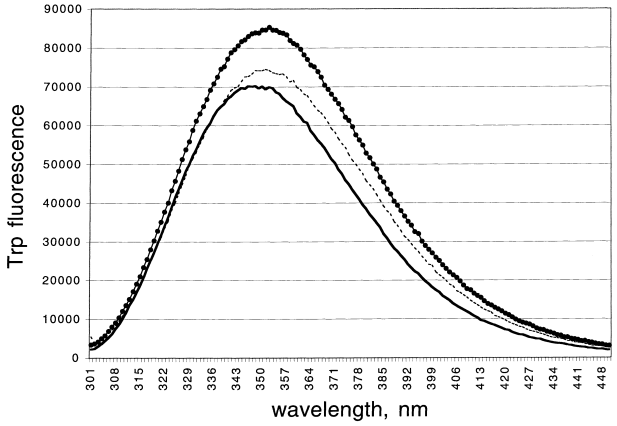

Fig. 3. Overlap between corrected fluorescence spectra of apo-S100A1, holo-S100A1 (calcium bound form) and heterocomplex S100A1/A4. Solid line, apo-S100A1; dotted line, holo-S100A1; dashed line, S100A1/S100A4 complex. All spectra were obtained at 20°C in 10 mM Tris–HCl pH 7.5, 100 mM KCl and 500 nM of S100A1. The excitation wavelength was 295 nm. The spectrum of holo-S100A1 was recorded in the presence of 1 mM $CaCl_2$. See the text for further details.

the average mass of 24 982.00 Da. This is in agreement with the average mass 24 981.61 Da that could be predicted from the individual monomer masses for a non-covalent heterodimeric complex between S100A1 and S100A4. Signals corresponding to non-covalent homodimeric complexes of both S100A1 and S100A4 were also observed, as indicated in Fig. 2.

The presence of the unique tryptophan residue (W90) in the C-terminus of S100A1 allowed us to apply the fluorescence spectrometry technique to monitor the S100A1–S100A4 interaction by changes in tryptophan fluorescence. Addition of calcium to 1 mM caused a red shift of the S100A1 fluorescence emission spectrum by 4 nm and an increase in the fluorescence intensity at 351 nm indicating that W90 became exposed to the solvent. Addition of saturating amounts of S100A4 resulted in a decrease in the intensity of the tryptophan emission and a slight blue shift suggesting that W90 moved towards a more hydrophobic environment (Fig. 3). In the absence of calcium, the quenching effect caused by S100A4 was not accompanied by a shift in the emission maximum of the single tryptophan of S100A (not shown). These observations suggest that the interaction occurs both in the presence and in the absence of calcium, however, the mechanisms of the S100A4/S100A1 complex formation are different.

4. Discussion

Two-hybrid screening of the CSML-100 mouse mammary adenocarcinoma library revealed that the metastasis-promoting S100A4 (Mts1) protein forms a heterocomplex with S100A1, another member of the S100 family. Mutation of the conservative residues Phe^{72} and Leu^{79} in S100A4 abolished both the homodimerization of S100A4 and the interaction with S100A1. This was expected since these residues are a part of the hydrophobic core and seem to be important for the integrity of the S100 proteins [23]. Surprisingly, the S100A4/S100A1 interaction was completely abolished when Cys^{76} or/and Cys^{81}in the S100A4 protein were replaced by Ser. This mutation had no effect on the S100A4 homodimerization, suggesting that the mechanism of the S100A4/S100A1 heterodimerization could be different from that of the homodimerization of S100A4. Dissociation of the S100A4/S100A1 complex into subunits in the presence of SDS under non-reducing conditions indicated that cysteine disulfides are not involved in the complex formation.

Our fluorescence spectrometry data suggest that in the presence of calcium the tryptophan residue in S100A1 becomes part of a hydrophobic cluster in the S100A1/S100A4 heterodimer but is exposed in the S100A1 homodimer. The heterodimerization does not absolutely require calcium. Further investigation is therefore necessary to understand how the S100 complexes are formed and assembled in the presence and in the absence of calcium. According to the recently published three-dimensional structures of the S100A6 [23] and S100B [24] proteins, monomer–monomer interfaces are dominated by interactions between helices I, I′, IV and IV′, whereas putative dimer–dimer interactions may occur via hydrophobic residues in the C-terminal domain, which are exposed in the presence of calcium. Gribenko and Makhatadze [25] suggested that in the S100P protein Cys^{85} and Tyr^{88}, which are located besides the helix IV, are possibly involved in the formation of oligomerization interface. Taking these observations together, we have to exclude a single, simple model. It is most likely that the intermolecular interaction of the S100 subunits requires two different recognition surfaces. One of these surfaces could be specifically designed for canonical dimer formation [23] and the other for the interaction with target molecules or for the formation of oligomers. It may well be that heterodimerization of S100 proteins requires formation of higher order oligomeric intermediates for the subunit exchange. Perhaps, the mutations $Cys^{76} \rightarrow Ser$ and/or $Cys^{81} \rightarrow Ser$ abolish the formation of such putative intermediates and therefore prevent heterodimerization of S100A4/S100A1.

Members of the Sl00 family are known to be multifunctional proteins. However, little is known about the mechanisms that regulate the diversity of the S100 functions. The heterodimerization seems to be one of such mechanisms as there are several examples where homo- and heterodimers of S100 demonstrate different biological effects. For instance, the S100A1/S100B ($\alpha\beta$) heterodimer has been shown to inhibit the assembly of brain microtubule proteins in the presence of zinc, while the S100A1 ($\alpha\alpha$) homodimer had no such effect [26]. Another example of the S100 heterodimerization is the complex formed by S100A8 (calprotectin or MRP8) and S100A9 (MRP14). Newton and Hogg demonstrated that although the S100A9 protein stimulates neutrophil adhesion mediated by β-integrin Macl, the adhesion could be inhibited by S100A8 through the formation of the S100A8/S100A9 heterodimer [27]. The heterodimeric complex between S100B and S100A6 has recently been reported by Yang et al. [28]. The heterodimerization was demonstrated by the yeast two-hybrid system and confirmed by immunoprecipitation from melanoma cell lysates. However, the biological effects of this interaction are not known.

We provide here the first evidence for the interaction between S100A1 and Sl00A4 (Mts1). The biological significance of this interaction remains to be determined. We have found that S100A4 and S100A1 are co-expressed in several analyzed mouse adenocarcinoma cell lines (not shown), indicating that there is a probability that the S100A4/S100A1 heterodimerization may have a functional meaning. One can hypothesize

that the heterodimer can have cellular targets different from those of the S100A4 and S100A1 homodimers.

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