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Generation and characterization of a novel, permanently active S100P mutant

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ABSTRACT

S100 proteins function as Ca^{2+} signal transducers by regulating cellular targets in their Ca^{2+} bound conformation. S100P is a member of the S100 protein family that can activate the membrane and F-actin binding protein ezrin in a Ca^{2+} dependent manner at least in vitro. Here we generated a novel tool to elucidate directly the S100P–ezrin interaction in vivo. This was achieved by constructing a S100P derivative that contained mutations in the two EF hand loops predicted to lock the protein in a permanently active state. The resulting S100P mutant, termed here S100P pa, could be purified as a soluble protein and showed biochemical properties displayed by wild-type S100P only in the presence of Ca^{2+}. Importantly, S100P pa bound to the N-terminal domain of ezrin in the absence of Ca^{2+} showing an affinity only slightly reduced as compared to that of Ca^{2+}-bound WT S100P. In line with this permanent complex formation, S100P pa colocalized with ezrin to plasma membrane protrusions of epithelial cells even in the absence of intracellular Ca^{2+} transients. Thus, S100P pa is a novel type of S100 protein mutant locked in a permanently active state that shows an unregulated complex formation with its cellular target ezrin.

1. Introduction

Cellular Ca^{2+} transients can be evoked by a wide range of extracellular and intracellular stimuli. They serve important second messenger functions by initiating signaling pathways that result in a variety of responses ranging from altered gene expression to cell shape changes, cell migration and apoptosis. Many proteins coupling the initial Ca^{2+} signal to the downstream responses have been described. Most of these proteins are directly regulated by fluctuations in cellular Ca^{2+} as they harbor Ca^{2+} binding sites of different affinities. One class of Ca^{2+} binding proteins is characterized by the EF hand, a helix–loop–helix motif in which side chain and carbonyl oxygens coordinate the Ca^{2+} ion. S100 proteins form the largest group of EF hand proteins. They are short polypeptides of appr. 10 kDa and contain two EF hands per molecule. Whereas the N-terminal EF hand deviates slightly from the classical fold and has a rather low affinity, the C-terminal EF hand of S100 proteins is considered canonical and typically has a Ca^{2+} affinity in the micromolar range. S100 proteins form homo- and heterodimers and in most cases these dimers constitute the physiologically active unit (for reviews see [1–3]).

Ca^{2+} binding induces conformational changes in S100 proteins that typically are characterized by a reorientation of helix E2 (the E helix of the second EF hand) and result in the exposure of hydrophobic surfaces often involving residues found in the linker region between the two EF hands and the C-terminal extension following the second EF hand (for review see [4,5]). The Ca^{2+} bound conformation enables S100 proteins to bind to a large number of targets (effectors) occurring both intra- as well as extracellularly (for reviews see [2,3]). Within the S100 family, S100A10 represents an exception since it has suffered amino acid deletions and substitutions in both EF hands that abolish Ca^{2+} binding and lock the protein in a conformation resembling that of an active, Ca^{2+} bound S100 protein. Hence, S100A10 is able to form a tight, heterotetrameric complex with its target, annexin A2, even in the absence of Ca^{2+} [6–8].

S100P is a member of the S100 family initially identified in placenta [9]. It is expressed in a number of cells and tissues and is significantly upregulated in highly metastatic cancer cells suggesting an involvement in tumor cell migration [10–12]. Upon Ca^{2+} binding, S100P can interact with ezrin, a membrane-actin crosslinking protein. Ezrin is a member of the ERM (ezrin–radixin–moesin) family of proteins that are characterized by two principal domains, the N- and C-terminal ERM association domains (ERMADs). In resting cells N- and C-ERMAD interact with one another thereby masking the membrane protein and lipid binding sites located in the N-ERMAD and the F-actin binding site located in the C-ERMAD (for review see [13]). S100P can bind to the N-ERMAD in masked or dormant ezrin and this interaction activates ezrin's F-actin binding [14]. The S100P–ezrin interaction is strictly Ca^{2+} dependent in vitro and also appears to occur in vivo in stimulated cells upon intracellular Ca^{2+} elevation. Moreover, experiments with Ca^{2+} ionophores suggest that

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intracellular Ca^{2+} rises can directly activate the membrane-cytoskeleton crosslinking function of ezrin bypassing the need of protein phosphorylation [14]. Together, these observations have fed the idea that intracellular Ca^{2+} elevation results in Ca^{2+} binding to S100P and that this Ca^{2+}-bound S100P can in turn activate the ezrin molecule inducing its translocation to the membrane cytoskeleton. However, it is not known whether Ca^{2+} bound S100P is indeed an important mediator in this order of events within cells.

To directly address this question we have constructed a S100P mutant that should be locked in the active, Ca^{2+} bound conformation. Structural predictions indicate that the three-dimensional fold of this mutant, termed S100P pa for permanently active, resembles that of Ca^{2+} loaded S100P. In vitro analyses reveal that S100P pa shows biochemical properties that are displayed by wild-type S100P only in the presence of Ca^{2+}. Furthermore, S100P pa colocalizes with ezrin to membrane protrusions and microvilli of resting cells whereas WT S100P shows this property only after cell stimulation and intracellular Ca^{2+} rise. This indicates that the S100P–ezrin interaction is strictly dependent on the Ca^{2+} bound conformation of S100P also in vivo. The S100P pa derivative described here constitutes the first engineered permanently active mutant among the S100 proteins and is expected to serve as a useful tool in functional analyses of this and, by extrapolation, other S100 proteins.

2. Materials and methods

2.1. Construction of plasmids

The cloning of pET28a+-, pEGFP-C2- and pGEX4T-1 constructs encoding N-terminally histidine- or GFP-tagged human S100P WT, as well as histidine- or GST-tagged ezrin's N-ERMAD (aa1–323) had been described previously [14,15]. cDNA encoding the human S100P pa mutant was generated by polymerase chain reactions using the pET28a+ S100P WT plasmid as template and mutated oligonucleotide primers containing the respective nucleotide-substitutions and -deletions. For bacterial protein expression of His-tagged S100P WT, S100P pa and N-ERMAD the respective cDNAs were cloned into pET28a+ (Novagen) in frame with the 6× His-tag and, in case of GST-tagged N-ERMAD, into the pGEX4T-1 vector (Amersham Bioscience) in frame with the GST-tag. For the recombinant expression of non-tagged proteins the cDNA of the S100P pa mutant was cloned into a pET23a+ vector (Novagen, Madison, WI) modified to express the recombinant protein without T7 tag and, in the case of S100P WT, into the pKK223-3 vector (Amersham Pharmacia Biotech Freiburg, Germany). For localization analyses coding sequences of S100P derivatives were cloned into the pEGFP-C2 vector (Clontech, Mountain View, USA) to yield in frame fusion proteins with an N-terminal EGFP.

2.2. Cell culture

The human epidermoid carcinoma cell line A-431 was cultured in RPMI 1640 medium (PAA, Linz, Austria) supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum and antibiotics.

2.3. Transient transfection

A-431 cells were transiently transfected by nucleofection using Nucleofector Kit T (Amaxa, Köln, Germany) and the recommended program according to manufacturer's instructions. Following transfection A-431 cells were seeded on cover slips and cultured for 24 h.

2.4. Immunofluorescence staining

To induce cell stimulation accompanied by an intracellular Ca^{2+} mobilization transiently transfected A-431 cells grown on cover slips were transferred to growth media without FCS and cultured for 16 h. 10% heat-inactivated fetal bovine serum was then added and cells were incubated for 30 min. Control non-stimulated cells were maintained in FCS-free serum. Subsequently, cells were fixed with 3% formaldehyde in PBS for 10 min at room temperature. After quenching in 50 mM NH_4Cl for 7 min cells were permeabilized with 0.2% Triton X-100 in PBS for 2 min and incubated in PBS supplemented with 2% BSA for 30 min. Cells were then incubated with an anti-ezrin polyclonal antibody (Upstate, Lake Placid, USA) for 30 min and washed three times with 2% BSA/PBS, followed by staining of the primary antibody with Cy2-conjugated goat anti-rabbit secondary antibody (Dianova, Hamburg, Germany). Cells were washed three times with PBS, once with distilled H_2O, mounted in Mowiol and analyzed using a confocal laser scanning microscope (LSM 510; Zeiss, Jena, Germany).

2.5. Recombinant expression and purification of S100P WT, S100P pa and N-ERMAD

pET28a+-constructs encoding N-terminally His-tagged WT and mutant S100P, pET23a+ and pKK223-3 constructs encoding S100 derivatives without tag, as well as the pET28a+- and pGEX4T-1 constructs encoding ezrin's N-ERMAD with an N-terminal histidine- or GST-tag were used to transform *E. coli* [strain BL21 (DE3)pLysS]. Transformed bacteria were grown to an OD_{600} of 0.6 and recombinant protein expression was then induced by adding IPTG. After 3 h of incubation, cells were harvested by centrifugation (5000 × *g*, 10 min) and resuspended in lysis buffer (40 mM Hepes, pH 7.2, 300 mM NaCl, 20 mM imidazole, pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, 1.5 mM PMSF). Subsequently, cells were lysed by repeated freeze/thaw cycles (3 times) and sonication and the lysates were cleared by centrifugation for 1 h at 100,000 × *g*.

For preparation of His-tagged S100P WT the remaining supernatant was adjusted to a Ca^{2+} concentration of 5 mM and applied to a phenyl Sepharose (Pharmacia, Freiburg, Germany) column equilibrated in lysis buffer containing 0.5 mM $CaCl_2$ instead of EDTA. After extensive washing with the same buffer, bound protein was eluted with lysis buffer containing 1 mM EGTA. S100P-containing fractions were pooled, dialysed against buffer N (20 mM imidazole, pH 7.5, 300 mM NaCl, 10 mM β-mercaptoethanol, 1.5 mM PMSF), and applied to a Ni-NTA-agarose (Qiagen, Hilden, Germany) column equilibrated in the same buffer. After several washing steps with buffer N, S100P WT protein was eluted with 250 mM imidazole, pH 7.5, 300 mM NaCl, 10 mM β-mercaptoethanol and 1 mM PMSF.

For preparation of His-tagged S100P pa the cleared bacterial lysate was directly applied to a Ni-NTA-agarose column and purified by developing the column as described above for the His-tagged S100P WT protein.

For preparation of non-tagged S100P WT the cleared bacterial lysate was applied to a phenyl Sepharose column and purified by developing the column as described above for the His-tagged S100P proteins.

The non-tagged S100P pa protein was purified using anion exchange chromatography. Therefore, the cleared bacterial lysate was first dialysed against buffer D (10 mM Hepes pH 7.2, 20 mM NaCl, 10 mM 2-mercaptoethanol, 1 mM PMSF) and then applied to a DEAE-Sephacel (GE-Healthcare) column equilibrated in buffer D. After extensive washing with buffer D, S100P pa protein was eluted with 10 mM Hepes pH 7.2, 500 mM NaCl, 10 mM 2-Mercaptoethanol and 1 mM PMSF.

The histidine-tagged N-ERMAD protein was purified as described [14].

In the case of GST-tagged N-ERMAD the cleared bacterial lysate was applied to a glutathione Sepharose 4B (Amersham Biosciences, Uppsala, Sweden) column equilibrated in PBS containing 1.5 mM PMSF. After extensive washing with the same buffer, bound N-ERMAD protein was eluted with 100 mM glutathione, 50 mM Tris–HCl, pH 8.0.