Interleukin-11 binds specific EF-hand proteins via their conserved structural motifs

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Interleukin-11 (IL-11) is a hematopoietic cytokine engaged in numerous biological processes and validated as a target for treatment of various cancers. IL-11 contains intrinsically disordered regions that might recognize multiple targets. Recently, we found that aside from IL-11RA and gp130 receptors, IL-11 interacts with calcium sensor protein S100P. Strict calcium dependence of this interaction suggests a possibility of IL-11 interaction with other calcium sensor proteins. Here we probed specificity of IL-11 to calcium-binding proteins of various types: calcium sensors of the EF-hand family (calmodulin, S100B and neuronal calcium sensors: recoverin, NCS-1, GCAP-1, GCAP-2), calcium buffers of the EF-hand family (S100G, oncomodulin), and a non-EF-hand calcium buffer (α-lactalbumin). A specific subset of the calcium sensor proteins (calmodulin, S100B, NCS-1, GCAP-1/2) exhibits metal-dependent binding of IL-11 with dissociation constants of 1–19 μM. These proteins share several amino acid residues belonging to conservative structural motifs of the EF-hand proteins, ‘black’ and ‘gray’ clusters. Replacements of the respective S100P residues by alanine drastically decrease its affinity to IL-11, suggesting their involvement into the association process. Secondary structure and accessibility of the hinge region of the EF-hand proteins studied are predicted to control specificity and selectivity of their binding to IL-11. The IL-11 interaction with the EF-hand proteins is expected to occur under numerous pathological conditions, accompanied by disintegration of plasma membrane and efflux of cellular components into the extracellular milieu.

Keywords: cancer; intrinsic disorder; interleukin; EF-hand; S100 protein; neuronal calcium sensor; protein–protein interaction

Introduction

A hematopoietic cytokine interleukin-11 (IL-11) is predominantly expressed by leukocytes, fibroblasts, epithelial cells, and exhibits numerous activities, such as stimulation of thrombopoiesis, erythropoiesis, lymphopoiesis, megakaryocytopoiesis, osteoclastogenesis, polarization of T cells, regulation of macrophage phagocytosis, stem cell development, and embryo implantation (Dimitriadis & Menkhorst, 2011; Ernst & Putoczki, 2014; Putoczki & Ernst, 2015; Yuzhalin & Kutikhin, 2015). The polyfunctionality of IL-11 suggests that it has multiple targets, which is typical for intrinsically disordered proteins with unordered regions able to attain defined structure upon target binding (Habchi, Tompa, Longhi, & Uversky, 2014). Indeed, the IL-11 molecule has two disordered regions (PDB: 4MHL (Putoczki, Dobson, & Griffin, 2014)) that could contribute to target recognition (see Figure 1(A)). This is further illustrated by Figure 2(A) that represents the results of the evaluation of the human IL-11 (UniProt ID: P20809) predisposition for intrinsic disorder by several computational tools and clearly shows that this protein is predicted to have functional disordered regions involved in interaction with natural binding partners. Figure 2(B) shows an interaction network of human IL-11 obtained using the STRING database (Szklarczyk et al., 2011) and supports the notion that this protein is a promiscuous binder.

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IL-11 normally binds to a cell-type specific IL-11 receptor subunit α (IL-11RA), followed by association with ubiquitously expressed interleukin-6 receptor subunit β (gp130) and downstream activation of several protumorigenic signaling pathways (Putoczki & Ernst, 2015). Increased IL-11 signaling is observed in numerous cancers and is associated with metastasis and poor prognosis (Onnis, Fer, Rapisarda, Perez, & Melillo, 2013; Putoczki & Ernst, 2015). IL-11 was also shown to bind oncoproteins MAGEA11 (Rolland et al., 2014) and S100P (Kazakov et al., 2015). The main intracellular location of these proteins (Bai, He, & Wilson, 2005) prevents their interaction with IL-11, but S100P colocalizes with IL-11 in blood (Wu et al., 2015). Remarkably, S100P-IL-11 interaction demonstrates strict calcium dependence, likely due to the calcium-induced solvent exposure of hydrophobic residues of S100P (Becker, Gerke, Kube, & Weber, 1992; Lee et al., 2004). The Ca\(^{2+}\)-induced solvation of hydrophobic regions is utilized by many calcium sensor proteins for target binding (Clapham, 2007). Hence, this process could favor interaction of the calcium sensors with hydrophobic residues nearby the unordered regions of IL-11. To explore this possibility and to evaluate selectivity and specificity of these interactions, we studied IL-11 interaction with Ca\(^{2+}\)-binding proteins belonging to several groups, including calcium sensors of the EF-hand family (calmodulin, S100B and neuronal calcium sensors: Rec, NCS-1, and GCAP-2 (Ames & Lim, 2012; Clapham, 2007; Lim, Dizhoor, & Ames, 2014; Zimmer & Weber, 2010)), calcium buffers of the EF-hand family (S100G, O\(\alpha\)M (Henzl & Tanner, 2007; Schwallier, 2009; Skelton, Kördel, & Chazin, 1995)), and a non-EF-hand calcium buffer (\(\alpha\)-lactalbumin (Chrysina, Brew, & Acharya, 2000)). A canonical EF-hand domain is observed throughout all of the domains of life and represents a helix-loop-helix structure, where a calcium-binding loop is flanked by two \(\alpha\)-helices (see Figure 1(B)). The revealed binding of IL-11 to certain calcium sensors of the EF-hand family and underlying structural regularities of that process opens new perspectives for search of other possible interaction partners of this pleiotropic cytokine upon pathological conditions accompanied by disintegration of plasma membrane.

**Materials and methods**

**Materials**

Recombinant human IL-11 (rWT IL-11) and human S100P were prepared as previously described (Kazakov et al., 2015). Calmodulin isolated from bovine brain (CaM; amino acid sequence is identical to that for the human protein) according to Pernyakov, Shnyrov, Kalinichenko and Orlov (1985) was kindly provided by Dr. Nicolay Ya. Orlov (ITEB RAS, Pushchino, Russia). Recombinant forms of myristoylated bovine Rec and neuronal calcium sensor 1 (NCS-1; 99% sequence identity to the human protein) were prepared as described in Baksheeva et al. (2015). Recombinant forms of myristoylated bovine GCAP-1 and 2 (GCAP-1/2) were obtained according to Hwang and Koch (2002) and Zernii et al. (2015) (the genetic constructs were kindly provided by Prof. Karl-Wilhelm Koch, University of Oldenburg, Germany). Intact rat \(\alpha\)-parvalbumin (PV) was isolated as described in Pernyakov et al. (2012). \(\alpha\)-Lactalbumin from bovine milk (BLA) and albumin from human serum (HSA) were purchased from Sigma-Aldrich (cat. #61,289 and #A3782, respectively). Protein concentrations were measured...
Figure 2. (A) The per-residue disorder propensity of the human IL-11 (UniProt ID: P20809) evaluated by members of the PONDR family, PONDR® VLXT (black line), PONDR® VSL2 (blue line), PONDR® FIT (dark pink line), and PONDR® VL3 (red line). Localization of known α-helices of this protein is shown by light gray shaded areas. Light pink shadow around the PONDR® FIT curve represents distribution of errors in the disorder score evaluation. In this plot, residues and regions with scores above .5 are considered as disordered. PONDR® VSL2B is one of the most accurate stand-alone disorder predictors (Obradovic, Peng, Vucetic, Radivojac, & Dunker, 2005), PONDR® VL3 possesses a high accuracy for finding long disordered regions (Obradovic et al., 2003), PONDR® VLXT is not the most accurate predictor but has a high sensitivity to local sequence peculiarities which are often associated with disorder-based interaction sites (Dunker et al., 2001), whereas PONDR-FIT represents a metapredictor which, due to being moderately more accurate than each of the component predictors, is one of the most accurate disorder predictors (Xue, Dunbrack, Williams, Dunker, & Uversky, 2010). Location of a potential disorder-based binding site identified by the ANCHOR algorithm (Dosztányi, Meszaros, & Simon, 2009; Meszáros, Simon, & Dosztányi, 2009) is shown as thick green bar (AIBS for ANCHOR-indicated binding site). This approach relies on the pairwise energy estimation approach developed for the general disorder prediction method IUPred (Dosztányi, Csizmok, Tompa, & Simon, 2005a, 2005b), being based on the hypothesis that long regions of disorder contain localized potential binding sites that cannot form enough favorable intrachain interactions to fold on their own, but are likely to gain stabilizing energy by interacting with a globular protein partner (Dosztányi et al., 2009; Meszáros et al., 2009). (B) Analysis of the interactivity of the human IL-11 (UniProt ID: P20809) by STRING (Szklarczyk et al., 2011). STRING produces the network of predicted associations for a particular group of proteins. The network nodes are proteins, whereas the edges represent the predicted or known functional associations. An edge may be drawn with up to seven differently colored lines that represent the existence of the seven types of evidence used in predicting the associations. A red line indicates the presence of fusion evidence; a green line – neighborhood evidence; a blue line – co-occurrence evidence; a purple line – experimental evidence; a yellow line – text mining evidence; a light blue line – database evidence; a black line – co-expression evidence (Szklarczyk et al., 2011).
spectrophotometrically using molar extinction coefficients at 280 nm calculated according to Pace, Vajdos, Fee, Grimsley and Gray (1995): 17,990 M−1 cm−1 (rWT IL-1), 2980 M−1 cm−1 (S100P and its mutants), 1490 M−1 cm−1 (S100B), 2980 M−1 cm−1 (S100G), 2980 M−1 cm−1 (CaM), 23,950 M−1 cm−1 (Rec), 21,430 M−1 cm−1 (NCS-1), 26,930 M−1 cm−1 (GCAP-1), 34,950 M−1 cm−1 (GCAP-2), 2980 M−1 cm−1 (OM), 28,460 M−1 cm−1 (BLA), 34,445 M−1 cm−1 (HSA). Molar extinction coefficient of PV at 258 nm of 1600 M−1 cm−1 (Permyakov et al., 2012) was used.

DEAE Sephacel, SP Sepharose Fast Flow and PD MidiTrap G-25 were purchased from GE Healthcare. Sephadex G-25 was a product of Pharmacia LKB. Toyopearl SuperQ-650M was from Tosoh Bioscience LLC. ProteOn™ GLH Sensor Chip and Amine Coupling Kit were from Bio-Rad Laboratories, Inc.

Preparation of S100P mutants

Alanine scanning of the residues comprising black (F15, F71, F74) and gray (K30, L33, L58) clusters (Denesiuk, Permyakov, Denesyuk, Permyakov, & Johnson, 2014) of human S100P was performed. The pET-11a plasmid encoding wild-type human S100P (kindly provided by Dr. Roger Barraclough, University of Liverpool, UK) was subjected to site-directed mutagenesis with buffer B. S100G was eluted with a linear gradient of PV at 28,000 × g for 40 min at 4°C, followed by precipitation with 40% ammonium sulfate (stirring in ice-water bath for 30 min) and centrifugation at 25,000 × g for 30 min at 4°C. The supernatant was dialyzed at 4°C against distilled water and pH adjusted using 3 M sodium acetate, pH 4.0 buffer to pH 4.0. The solution was loaded onto SP Sepharose Fast Flow column (.9 cm × 7.5 cm) equilibrated with 10 mM sodium acetate, pH 4.0 (buffer A). The column was washed with buffer A. S100G was eluted with 100 mM Tris-HCl, pH 8.0 buffer (flow rate of 2 mL/min). The fractions containing S100G were identified by SDS-PAGE, collected, dialyzed at 4°C against water, followed by dialysis against 20 mM MES-NaOH, pH 6.5 (buffer B). The dialyzed solution was loaded onto a DEAE Sephacel column (2 cm × 13 cm) equilibrated with buffer B. S100G was eluted with a linear gradient of NaCl (0–200 mM) in buffer B (flow rate of 3 mL/min). The purified protein was exhaustively dialyzed at 4°C against distilled water, freeze-dried and stored at −18°C. The absence of Met1 residue in S100G was confirmed by ESI-MS.

Preparation of human S100B

Rat S100B gene in pET-11 plasmid (kindly provided by Prof. David J. Weber, University of Maryland School of Medicine, USA) was converted into human S100B gene (Swiss-Prot: P04271) by E63N and S79A substitutions. The resulting gene was cloned into pET-11a vector between the Ndel and BamHI restriction sites. S100B expression and purification were performed according to the procedure described for human S100P (Kazakov et al., 2015), except for use of 2-mM EGTA during the protein elution from Phenyl Sepharose 6 Fast Flow column. The presence of Met1 residue in S100B was confirmed by ESI-MS.

Preparation of S100G

Gene of mouse S100G with C2S substitution was codon optimized for the expression in E. coli and cloned into pET-29a vector between the Ndel and Xhol restriction sites. The plasmid was transformed into E. coli strain BL21(DE3)-RIL. The cells were grown at 37°C in 2YT medium with 50 μg/mL kanamycin and .4% D-glucose, until absorbance at 600 nm reached .5 AU. S100G expression was induced by 1 mM IPTG. The cells were grown for 3.5 h, harvested by centrifugation at 5000 × g for 15 min at 4°C, resuspended in lysis buffer (50 mM Tris-HCl, 20 mM NaCl, 2 mM EDTA, 1 mM PMSF, 1 mM DTT, pH 7.5), and disintegrated using a French press. The lysate was centrifuged at 28,000 × g for 40 min at 4°C, followed by precipitation with 40% ammonium sulfate (stirring in ice-water bath for 30 min) and centrifugation at 25,000 × g for 30 min at 4°C. The supernatant was dialyzed at 4°C against distilled water and pH adjusted using 3 M sodium acetate, pH 4.0 buffer to pH 4.0. The solution was loaded onto SP Sepharose Fast Flow column (.9 cm × 7.5 cm) equilibrated with 10 mM sodium acetate, pH 4.0 (buffer A). The column was washed with buffer A. S100G was eluted with 100 mM Tris-HCl, pH 8.0 buffer (flow rate of 2 mL/min). The fractions containing S100G were identified by SDS-PAGE, collected, dialyzed at 4°C against water, followed by dialysis against 20 mM MES-NaOH, pH 6.5 (buffer B). The dialyzed solution was loaded onto a DEAE Sephacel column (2 cm × 13 cm) equilibrated with buffer B. S100G was eluted with a linear gradient of NaCl (0–200 mM) in buffer B (flow rate of 3 mL/min). The purified protein was exhaustively dialyzed at 4°C against distilled water, freeze-dried and stored at −18°C. The absence of Met1 residue in S100G was confirmed by ESI-MS.

Preparation of rat OM

The pET-11 plasmid encoding rat OM was kindly provided by Prof. Michael T. Henzl (University of Missouri, USA). The OM gene was cloned into pET-28a vector between the BantHI and Xbal restriction sites. The resulting plasmid was transformed into E. coli strain BL21(DE3)-RIL. The cells were grown at 37°C in 2YT medium with 50 μg/mL kanamycin A and .4% D-glucose until absorbance at 600 nm reached .6 AU. OM expression was induced by 1 mM IPTG. The cells were grown at 22°C for 4.5 h., harvested by centrifugation at 5,000 × g for 15 min at 4°C, resuspended in lysis buffer (50 mM Tris-HCl, 1 mM PMSF, 1 mM DTT, pH 7.4), and disintegrated using a French press. The lysate was centrifuged at 20,000 × g for 30 min at 4°C. 2 mM CaCl2 was added to the supernatant, followed by incubation at 80°C for 3 min, transferred into ice-water bath for 30 min, and centrifugation at 15,500 × g for 30 min at 4°C. The supernatant was dialyzed at 4°C against distilled water with 1 mM 2-ME, and once against 20 mM HEPES-KOH, 100 mM NaCl, 1 mM 2-ME,
pH 7.4 (buffer A). The solution was loaded onto DEAE Sephacel column (3 cm × 12 cm) equilibrated with buffer A. The column was washed with buffer A. OM was eluted with a linear gradient of NaCl (1.2–3 M) in buffer A without 2-ME (flow rate of 1.2 mL/min). The fractions containing OM were collected and dialyzed at 4°C against distilled water with 1 mM 2-ME, and once against 20 mM Tris-HCl, 150 mM KCl, 1 mM EDTA, pH 8.5 (buffer B). The dialyzed solution was loaded onto a Toyopearl SuperQ-650 M column (0.9 cm × 10 cm) equilibrated with buffer B without 2-ME (same buffer). OM was eluted with 20 mM Tris-HCl, EDTA and 2-ME. The column was washed with the same buffer. OM was eluted with 20 mM Tris-HCl, 60 mM CaCl2, pH 8.5 buffer (flow rate of 1 mL/min). The purified OM was exhaustively dialyzed at 4°C against distilled water with 1 mM 2-ME, and stored at −70°C. 60% of the OM sample lacks Met1 residue, as evidenced by ESI-MS (OM was preliminary reduced by 5 mM DTT, followed by passage through a PD MidiTrap G-25 column equilibrated with distilled water).

Surface plasmon resonance studies
Surface plasmon resonance (SPR) measurements were performed at 25°C using Bio-Rad ProteOn™ XPR36 protein interaction array system. Ligand (40 μg/mL rWT IL-11 in 10 mM sodium acetate, pH 4.5 buffer) was immobilized on ProteOn GLH sensor chip surface (up to 10,000 resonance units, RUs) by amine coupling, according to the manufacturer’s instructions. The remaining activated amine groups on the chip surface were blocked by 1 M ethanolamine solution. Analyte (S100P and its mutants, S100B, S100G, CaM, Rec, NCS-1, GCAP-1/2, PV, OM, BLA, HSA, 25 nM to 40 μM) in a running buffer (10 mM HEPES, 150 mM NaCl, .01% TWEEN 20, pH 7.4 buffer with either 1 mM CaCl2 and 2 mM MgCl2 (Ca2+-bound protein), or 1 mM EGTA and 2 mM MgCl2 (Mg2+-bound protein), or 1 mM EGTA (apo-protein)) was flowed over the chip at rate of 30 μl/min for at least 350 s, followed by flushing the chip with the running buffer for 1500 s. The double-referenced SPR sensograms were globally fitted according to a heterogeneous ligand model, which assumes existence of two populations of the ligand (L1 and L2) that bind single analyte molecule (A):

\[
\begin{align*}
K_{d1} & & K_{d2} \\
L_1 + A &\rightarrow& L_1A &\quad (1) \\
k_{d1} & & k_{d2}
\end{align*}
\]

where \(K_{d}\) and \(k_{d}\) refer to equilibrium and kinetic dissociation constants, respectively. Alternatively, the sensograms were globally fitted according to a 1:1 binding model:

\[
K_d \\
L + A \rightarrow LA \\
k_d
\]

\(K_{d}, k_{d}, \text{ and } R_{max}\) (maximum response) values were evaluated using Bio-Rad ProteOn Manager v.3.1 software. The sensor chip surface was regenerated by passage of .5% SDS water solution for 50 s.

Circular dichroism measurements
S100P and its mutants (2.6 mM) in 10 mM glycine-KOH, 5 mM EDTA, pH 9.2 buffer were decalcified by passage (.3 mL; .25 mL/min) through a Sephadex G-25 column (1 cm × 20 cm) equilibrated with deionized water. Far-UV circular dichroism (CD) spectra of the protein solution (10 μM) in 10 mM H3BO3, 1 mM EDTA, pH 8.8 buffer were measured at 15°C using JASCO J-810 spectropolarimeter as described in Permyakov et al. (2012). The spectra were analyzed in 200–240 nm range using CDPro software package (Sreerama, Venyaminov, & Woody, 2000).

Analysis of tertiary structures
Comparative analysis of protein structures was performed using BIOVIA Discovery Studio software (Accelrys). Analysis of protein/protein contacts and interfaces and identification of interacting substructures were done using Contacts of Structural Units (CSU) software, based on the surface complementarity approach (Sobolev, Sorokine, Prilusky, Abola, & Edelman, 1999).

Results
Metal-dependent interaction between IL-11 and specific EF-hand proteins
rWT IL-11 was immobilized on the surface of SPR sensor chip by amine coupling and a set of injections of analyte solutions (S100P, S100B, S100G, CaM, Rec, NCS-1, GCAP-1, GCAP-2, PV, OM, BLA, and HSA, in the presence/absence of Ca2+/Mg2+) was carried out. No changes in the SPR signal were observed for S100G (2.5–40 μM), PV (20–40 μM), OM (20–40 μM), BLA (20–500 μM), and HSA (20–40 μM), regardless of metal conditions (some data for S100G and Rec are shown in Figure 3). Analogous to S100P protein, the SPR sensograms for S100B and CaM (2.5–40 μM) exhibited a concentration-dependent association-dissociation pattern (Figure 3(A)) only under Ca2+ excess. Meanwhile, NCS-1, GCAP-1, and GCAP-2 (2.5–40 μM) associated with IL-11 both in the Ca2+-bound (Figure 3(B)) and Mg2+-bound (Figure 3(C)) states, but lacked observable effects in their apo-states (data not shown). The resulting kinetic SPR data were approximated by the heterogeneous

\[
K_{d1} \quad \frac{L_1 + A \rightarrow L_1A}{k_{d1}} \quad K_{d2} \quad \frac{L_2 + A \rightarrow L_2A}{k_{d2}}
\]
Figure 3. Kinetics of the interaction between rWT IL-11 and various EF-hand proteins at 25°C, monitored by SPR spectroscopy using IL-11 as a ligand. (A) Ca²⁺-bound forms of S100P (40 μM), S100B (40 μM), S100G (20 μM), and CaM (2.5 μM). (B) Ca²⁺-bound forms of NCS-1 (40 μM), GCAP-1 (40 μM), GCAP-2 (20 μM), and Rec (40 μM). (C) Mg²⁺-bound forms of NCS-1 (20 μM), GCAP-1 (20 μM), GCAP-2 (20 μM), and Rec (20 μM). 10 mM HEPES, 150 mM NaCl, 2 mM MgCl₂, .01% TWEEN 20, pH 7.4 buffer with either 1 mM CaCl₂ (Ca²⁺-bound protein) or 1 mM EGTA (Mg²⁺-bound protein). Gray curves are experimental, while black curves are theoretical, calculated according to either the heterogeneous ligand model (1) or the 1:1 binding model (2) (see Table 1 for the fitting parameters).

Table 1. Parameters of the heterogeneous ligand model (1), describing the SPR data on kinetics of interaction between rWT IL-11 and various EF-hand proteins (see Figure 3). Graphical representation of the $K_d$ values is shown in Figure 4.

| Analyte/parameter | Ca²⁺/Mg²⁺-bound protein | $k_{d1}$ (s⁻¹) | $K_{d1}$ (M) | $R_{max1}$ | $k_{d2}$ (s⁻¹) | $K_{d2}$ (M) | $R_{max2}$ |
|-------------------|-------------------------|----------------|-------------|------------|----------------|-------------|------------|
| S100P*            | Ca²⁺                    | (2.01 ± 0.1) × 10⁻⁴ | (3.2 ± 0.3) × 10⁻⁸ | 34 | (3.48 ± 0.02) × 10⁻³ | (2.88 ± 0.01) × 10⁻⁷ | 91 |
| S100B             | Ca²⁺                    | (1.12 ± 0.14) × 10⁻³ | (6.1 ± 1.6) × 10⁻⁹ | 45 | (3.14 ± 0.04) × 10⁻² | (3.64 ± 0.16) × 10⁻⁵ | 101 |
| CaM               | Ca²⁺                    | (2.06 ± 0.03) × 10⁻³ | (1.9 ± 0.3) × 10⁻⁵ | 64 | (5.3 ± 0.3) × 10⁻² | (1.2 ± 0.3) × 10⁻⁴ | 192 |
| NCS-1             | Ca²⁺                    | (5.52 ± 0.16) × 10⁻⁴ | (1.02 ± 0.05) × 10⁻⁵ | 75 | (1.32 ± 0.04) × 10⁻² | (1.3 ± 0.3) × 10⁻⁴ | 321 |
| Mg²⁺              | (7.6 ± 5.3) × 10⁻³      | (1.3 ± 0.2) × 10⁻⁵ | 27 | (5.4 ± 0.5) × 10⁻¹ | (7.3 ± 0.5) × 10⁻⁴ | 20 |
| GCAP-1            | Ca²⁺                    | (6.9 ± 1.5) × 10⁻⁴b | (1.3 ± 0.5) × 10⁻⁶b | 13 | NA             | NA          | NA         |
| Mg²⁺              | (3.37 ± 0.01) × 10⁻⁴    | (7.7 ± 3.3) × 10⁻⁷ | 179 | (5.44 ± 0.04) × 10⁻³ | (1.58 ± 0.14) × 10⁻⁶ | 196 |
| GCAP-2            | Ca²⁺                    | (4.2 ± 0.8) × 10⁻⁴ | (2.3 ± 0.5) × 10⁻⁶ | 48 | (1.19 ± 0.03) × 10⁻² | (4.48 ± 0.14) × 10⁻⁶ | 33 |
| Mg²⁺              | (5.1 ± 0.6) × 10⁻⁴      | (2.5 ± 0.3) × 10⁻⁶ | 32 | (8.6 ± 0.2) × 10⁻³ | (6.2 ± 0.5) × 10⁻⁶ | 30 |

*Data taken from Kazakov et al. (2015).

*According to a 1:1 binding model (2).

NA, data not available.
ligand model (1), except for the data for Ca\(^{2+}\)-bound GCAP-1, which were described by the simplest 1:1 binding model (2) (Figure 3 and Table 1). The inability of the latter model to describe the interaction between IL-11 and most of the EF-hand proteins studied reflects a complex mode of this interaction. The heterogeneity of the ligand may arise due to existence in IL-11 molecule of the multiple binding sites or to presence of IL-11 molecules with affinity to the target suffered from the IL-11 immobilization process. In either case, the lowest dissociation constant for a complex between IL-11 and its target EF-hand protein, \(K_{d1}\), is a measure of affinity of the strongest site of IL-11. The \(K_{d1}\) values measured by SPR technique for the Ca\(^{2+}\)-bound proteins grow in the row S100P < GCAP-1 < GCAP-2 < S100B < NCS-1 < CaM (Figure 4(A)), from 32 nM to 19 \(\mu\)M (Table 1). The replacement of Ca\(^{2+}\) by Mg\(^{2+}\) does not affect the \(K_{d1}\) values for IL-11 interaction with NCS-1, GCAP-1, and GCAP-2. Overall, rWT IL-11 selectively binds Ca\(^{2+}/\)Mg\(^{2+}\)-bound states of a specific subset of the EF-hand proteins studied, being non-specific to their apo-forms and close relatives.

**Exploration of structural determinants of IL-11 interaction with EF-hand proteins**

Multiple sequence alignment of the EF-hand proteins studied here (see Supplementary Figure S1) reveals that the highest degree of conservation is observed in the regions of Ca\(^{2+}\)-binding loops of S100P. The most conserved residues of these regions mainly provide the side chains directly coordinating Ca\(^{2+}\) ion, but residues F15, L33, and F74 belong to the recently identified highly conserved structural motifs found in all families of the EF-hand proteins, named ‘black’ and ‘gray’ clusters (Denessiouk et al., 2014). These motifs provide a supporting scaffold for the DxDxDG Ca\(^{2+}\)-binding loop and contribute to hydrophobic core of the EF-hand domain (Figure 5(A)). The residues F15, F71, F74 and K30, L33, L58 of S100P constitute its black and gray clusters, respectively (Supplementary Figure S1). While F71 and K30 are located within the EF-loops, other residues of the clusters are nearby them. Since bulky hydrophobic residues of the clusters are likely to be involved into IL-11 binding, they were subjected to alanine scanning mutagenesis. Analysis of far-UV CD spectra of the resulting F15A, F71A, F74A, K30A, L33A, and L58A mutants (Supplementary Figure S2) using CDPro software (Sreerama et al., 2000) evidence a retention of predominance of \(\alpha\)-helices in apo-forms of the mutants (Table 2). The fraction of \(\alpha\)-helices, \(f_\alpha\), in the mutants is increased by 8–10% with regard to WT S1000P (\(f_\alpha\) of 54%, consistent with PDB entry 1OZO), except for L58A, exhibiting an increase in \(f_\alpha\) value by 2%. Nevertheless, the most noticeable structural changes induced in S100P by K30A substitution caused the least prominent decline in S100P affinity to rWT IL-11, as evidenced by SPR measurements (Figures 4, 6, and 7 and Table 3). Meanwhile, mutagenesis of other residues of the gray and black clusters was accompanied by lowering of S100P affinity to IL-11, which is nearly proportional to the mutation-induced increase in \(f_\alpha\) value, in the scale of free energy changes (Figure 7). The mutations in the black cluster cause the most drastic lowering of S100P affinity to IL-11 (by 2–3 orders of magnitude). The
example of K30A mutant indicates that the pronounced suppression of S100P-IL-11 interaction observed for other mutants may be not due to the mutagenesis-induced global rearrangements in S100P structure, but due to direct or allosteric involvement of these residues into binding of IL-11. Thus, the residues of gray and black clusters of S100P, except for K30 (least conservative of them), are shown to be engaged into interaction with IL-11. Meanwhile, analysis of solvent accessible surface area (SASA) of apo- and Ca\(^{2+}\)-bound states of S100P reveals (Supplementary Figure S3) that these residues do not solvate upon Ca\(^{2+}\)-binding and therefore unlikely to initiate the Ca\(^{2+}\)-induced binding of IL-11. The most noticeable Ca\(^{2+}\)-induced increase in SASA is observed in the hinge region of S100P (Supplementary Figure S3). This effect likely favors the interaction of Ca\(^{2+}\)-bound S100P with V domain of receptor for advanced glycation end products (RAGE): the complex is formed by S100P residues 36–50 (include the hinge region 39–50) and its C-terminal residues 81–86 and 88–94 (Figure 5(B)). The hinge region is crucial for target recognition by the other EF-hand proteins interacting with IL-11, including S100B, calmodulin, and NCS-1 (see Supplementary Table S1 for respective PDB

Table 2. The secondary structure fractions estimated from CD data for apo-forms of WT S100P and its mutants at 15°C using CDPro software (Sreerama et al., 2000) (see Supplementary Figure S2 for the original data).

| Protein   | α-Helices (%) | β-Strands (%) | Turns (%) | Unordered structure (%) |
|-----------|---------------|---------------|-----------|-------------------------|
| WT        | 54.0 ± 1.1    | 6.2 ± .6      | 13.7 ± .1 | 26.2 ± .1               |
| Black cluster |          |               |           |                         |
| F15A      | 63 ± 2        | 3.0 ± .5      | 11.1 ± .6 | 22.2 ± 1.0              |
| F71A      | 61.3 ± .2     | 5.0 ± .4      | 11.9 ± .7 | 22.0 ± 1.1              |
| F74A      | 61.6 ± 1.6    | 4.1           | 12.0 ± .7 | 21.9 ± 1.1              |
| Gray cluster |          |               |           |                         |
| K30A      | 64 ± 4        | 4.0 ± .8      | 10.9 ± 1.4| 22 ± 2                  |
| L33A      | 62.9 ± 1.8    | 3.8 ± .1      | 11.2 ± .5 | 22.4 ± 1.3              |
| L58A      | 56 ± 2        | 5.7 ± .4      | 13.1 ± .3 | 25.6 ± 1.0              |
entries). The hinge represents a common target recognition site in S100 proteins (Permyakov et al., 2011). Examination of tertiary structures of the EF-hand proteins studied here or their orthologs (see Supplementary Table S2) reveals that structures of their hinge regions may be classified into several states, differing by combination of two parameters: secondary structure of hinge region (contains helical structure or unordered) and presence of protein fragments, which shield the hinge region from possible interactions. The analyzed Ca$^{2+}$-bound forms of the proteins shown here to interact with IL-11 mainly demonstrate a loop structure of the hinge, which is accessible for interactions. Apo-states of these proteins and the proteins not interacting with IL-11 mostly exhibit either presence of helical structure in the hinge region (Figure 5(C)) or blocking of the hinge from interactions by nearby protein fragments, or both. The association of the Ca$^{2+}$-bound forms of the EF-hand proteins with their targets mainly does not affect the aforementioned parameters of the hinge region (compare the data in Supplementary Tables S1 and S2). Thus, calcium binding induces the hinge conformation favoring target binding. Overall, the structural features of the hinge region likely ensure selectivity and calcium sensitivity of IL-11 binding to the EF-hand proteins studied.

Discussion

The revealed ability of S100P, S100B, CaM, NCS-1, GCAP-1/2 to Ca$^{2+}$-dependent association with IL-11 is in accord with their calcium sensory properties: Ca$^{2+}$-induced solvation of the hydrophobic residues involved into target recognition (Ames & Lim, 2012; Becker et al., 1992; Lee et al., 2004; Lim et al., 2014; Zimmer & Weber, 2010) may promote their interaction with hydrophobic residues adjacent to the disordered regions of IL-11 molecule (PDB: 4MHL). The lack of well-defined solvated hydrophobic surfaces in the calcium buffer proteins studied, S100G, PV, OM, and BLA, regardless of Ca$^{2+}$ conditions (Chrysina et al., 2000; Henzl & Tanner, 2007, 2008;
Figure 7. The dependence of free energy changes, accompanying the interaction between rWT IL-11 and Ca\textsuperscript{2+}-bound forms of the S100P mutants, on the content of \(\alpha\)-helices in their apo-states. Refer to Figure 2 and Table 2 for the SPR and CD data used, respectively. The dashed lines are linear approximations of the experimental points, excluding the data for K30A mutant.

Table 3. Parameters of the heterogeneous ligand model (1), describing the SPR data on kinetics of interaction between rWT IL-11 and Ca\textsuperscript{2+}-bound forms of the S100P mutants (see Figure 6). Graphical representation of the \(K_d\) values is shown in Figure 4.

| Analyte/parameter | \(k_{d1}\) (s\(^{-1}\)) | \(K_{d1}\) (\(\mu\)M) | \(R_{max1}\) | \(k_{d2}\) (s\(^{-1}\)) | \(K_{d2}\) (\(\mu\)M) | \(R_{max2}\) |
|-------------------|-----------------|-----------------|-------------|-----------------|-----------------|-------------|
| WT\(^a\)          | \((2.01 \pm .01) \times 10^{-4}\) | .032 \pm .003 | 34          | \((3.48 \pm .02) \times 10^{-3}\) | .288 \pm .001 | 91          |
| Black cluster     |                 |                 |             |                 |                 |             |
| F15A              | \((6.6 \pm 1.1) \times 10^{-4}\) | 3.0 \pm .5     | 42          | \((8.9 \pm .9) \times 10^{-3}\) | 32 \pm 2     | 230         |
| F71A              | \((7.8 \pm .9) \times 10^{-3}\) | 2.3 \pm .2     | 23          | \((7 \pm 3) \times 10^{-4}\) | 23.4 \pm 1.7 | 225         |
| F74A              | \((2.00 \pm .14) \times 10^{-3}\) | 1.8 \pm .8     | 94          | \((3.13 \pm .03) \times 10^{-2}\) | 10.3 \pm 1.6 | 224         |
| Gray cluster      |                 |                 |             |                 |                 |             |
| K30A              | \((4.49 \pm .03) \times 10^{-4}\) | .117 \pm .004 | 151         | \((7.81 \pm .11) \times 10^{-3}\) | .41 \pm .09 | 10          |
| L33A              | \((2.01 \pm .03) \times 10^{-3}\) | .66 \pm .04    | 75          | \((3.77 \pm .03) \times 10^{-2}\) | 1.0 \pm .3   | 73          |
| L58A              | \((5.3 \pm .6) \times 10^{-4}\) | .32 \pm .02    | 230         | \((5.0 \pm .4) \times 10^{-3}\) | .79 \pm .15 | 223         |

\(^a\)Data taken from Kazakov et al. (2015).
Skelton et al., (1995), seems to prevent their binding to IL-11. Meanwhile, the absence of IL-11 interaction with Ca\(^{2+}\)-bound Rec, exhibiting an exposed hydrophobic groove intended for target binding (Ames & Lim, 2012; Lim et al., 2014) indicates that hydrophobicity of a protein is not the only factor governing the association with IL-11. Analysis of tertiary structures of the EF-hand proteins studied here points out that structural state of their hinge region controls their specificity to IL-11 and its calcium sensitivity. Considering low conservation degree of the hinge region among the EF-hand proteins interacting with IL-11 (Supplementary Figure S1), the hinge is unlikely to serve as a structural factor imparting to these related proteins the ability to bind IL-11. The latter could be achieved via conserved residues of the gray and black clusters of these proteins (as confirmed by the mutagenesis of S100P) along with some other residues, for example, those corresponding to the C-terminal S100P residues 81–86 and 88–94 interacting with RAGE (Figure 5(B)). One of them, residue 88, is a bulky hydrophobic residue present in the most EF-hand proteins shown to bind IL-11 (Supplementary Figure S1). It is directly involved into target binding also in S100B (PDB: 5CSI). Since this residue is absent in most of the proteins not interacting with IL-11 (Supplementary Figure S1), it could contribute into selectivity of this interaction.

Remarkably, cluster residues L33 and L58 (gray cluster), F71 and F74 (black cluster) of S100P are conserved in IL-11RA and gp130 receptors (Supplementary Figure S4). Furthermore, S100P residues V14, M37, L57, and Y88 are also conserved among these receptors and the EF-hand proteins studied (Supplementary Figure S4). Hence, the revealed specificity of certain EF-hand proteins to IL-11 may arise due to their structural similarity to some regions of IL-11RA and gp130 receptors. Analysis of location of the residues of these receptors contacting with IL-6 molecule in PDB entry 1P9 M (Supplementary Figure S4) shows that they mostly do not intersect with the aforementioned conservative residues (except for residue V14 of S100P), while many contacts of the receptors with IL-6 are located in the hinge region and canonical EF-loop of S100P (for IL-11RA). Thereby, the residues conserved in IL-11RA and gp130 receptors and in the EF-hand proteins could provide a structural basis for positioning of these loop regions favoring their interaction with IL-11. Nevertheless, structural comparison of these conservative regions of the receptors (PDB: 1P9 M) does not reveal evident structural similarities with the corresponding regions of the EF-hand proteins, which may imply that the suggestion on structural identity of the hexameric complexes of interleukins 6 and 11 with their receptors is invalid. Qualitatively, different modes of IL-11 interaction with IL-11RA and gp130 receptors could explain opposite functions of IL-6 and IL-11 under in vivo conditions (Garbers & Scheller, 2013).

The affinities of S100B and CaM to IL-11 (Table 1) are insufficient for their interaction in blood, since plasma concentrations of S100B, CaM, and IL-11 are within dozens of picomoles (Cai et al., 2011; Esteras et al., 2013; Ren, Chen, Han, Fu, & Chen, 2014). Meanwhile, high intracellular concentrations of CaM and S100B (reach dozens of micromoles (Donato, 1986; Kakiuchi et al., 1982)), NCS-1 (micromolar level in the nervous system (Schaad et al., 1996)) and GCAP-1/2 (micromolar level (Hwang et al., 2003)) open up the possibility of their local interaction with extracellular IL-11 in case of the processes, accompanied by disintegration of plasma membrane and efflux of cellular contents into the extracellular space. This is the case of necrosis in the course of Alzheimer disease, Creutzfeldt-Jakob disease, epilepsy, inflammatory, ischemic, and infectious diseases (Proskuryakov, Konoplyannikov, & Gabai, 2003). Myocardial infarction is accompanied with necrosis of cardiomyocytes (express CaM (Wu & Bers, 2007), NCS-1 (Nakamura et al., 2003) and S100B (Sorci et al., 2010) Kajstura et al., 1996). Stroke and traumatic brain injury are shown to release S100B into the extracellular milieu, cerebrospinal fluid, and blood (Saenger & Christenson, 2010; Sorci et al., 2010). The analogous effects are expected for CaM and NCS-1. Since prolonged intense illumination of retina leads to disintegration of the photoreceptor cells and release of Rec into the extracellular space (Zernii et al., 2015), the same effect likely occurs for GCAP-1/2 of the photoreceptors (Lim et al., 2014). The ability of nearby retinal pigment epithelial cells to IL-11 secretion (Nagineni, Komineni, William, Hooks, & Detrick, 2010) favors its association with the released GCAP-1/2. Similarly, the release of GCAP-1/2 might occur upon autoimmune uveitis, when exposed Rec serves as an ocular antigen (Papotto, Marengo, Sardinha, Goldberg, & Rizzo, 2014). The binding of the released EF-hand proteins by IL-11 could modulate its signaling in multiple ways, as previously described (Kazakov et al., 2015). The revealed structural regularities of the EF-hand proteins enabling their interaction with IL-11 might facilitate search of similar interaction partners of this cytokine and explain its pleiotropic properties.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| AIBS         | ANCHOR-identified binding site |
| BLA          | α-lactalbumin isolated from bovine milk |
| CaM          | calmodulin isolated from bovine brain |
| CD           | circular dichroism; CSU, Contacts of Structural Units software; |
| CSU          | contacts of structural units software |
| DTT          | DL-dithiothreitol |
EDTA ethylenediamine tetraacetic acid
EGTA ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
ESI-MS electrospray ionization mass spectrometry
GCAP-1/2 recombinant myristoylated bovine guanylyl cyclase-activating protein 1/2
gp130 interleukin-6 receptor subunit β
HEPES 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HSA albumin isolated from human serum
IL-6 interleukin-6
IL-6RA interleukin-6 receptor subunit α
IL-11 interleukin-11
IL-11RA interleukin-11 receptor subunit α
IL-11RA intercellular adhesion molecule-1
IPTG isopropyl β-D-1-thiogalactopyranoside
2-ME 2-mercaptoethanol
MES 2-(N-morpholino)ethanesulfonic acid
NCS-1 neuronal calcium sensor 1
OM recombinant rat oncomodulin
PMSF phenylmethanesulfonyl fluoride
PV α isoform of parvalbumin isolated from rat muscles
RAGE receptor for advanced glycation end products
Rec recombinant myristoylated bovine recoverin
RU resonance unit
rWT IL-11 recombinant human interleukin-11 omitting the N-terminal Pro residue of the mature protein (residues 23–199 of Swiss-Prot entry P20809)
SASA solvent accessible surface area
SPR surface plasmon resonance
S100G C2S mutant of mouse S100G
(SWISS-PROT entry P20809)
S100P/S100B recombinant human S100P/S100B

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**Supplementary material**

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**Author contributions**

VVG, TAM, EAP, and SEP conceived and supervised the study; ASK, KAD, AID, VNU, and SEP designed experiments; ASK, ASS, AAV, MEP, PAK, RGI, KAD, AID, WEB, EYZ, VNU, and DVZ performed experiments; ASK, KAD, AID, VNU, and SEP analyzed data; VAR, VNU, TAM, EAP, and SEP wrote the manuscript.
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