ABSTRACT

The immune system is the first line of defense in the fight against infections in the body caused by various pathogens. In the fight against cancer cells, therapy based on blocking immune checkpoints seems promising. Receptors and their ligands that are capable of regulating the activation of the immune system are called immune checkpoints. The formation of the receptor/ligand complex provides signals that stimulate or inhibit T cell activation, allowing the immune system to maintain a state of equilibrium. One example of such complex is the interaction of the CD160 receptor protein with the HVEM protein, which is located on the surface of cancer cells. The formation of the HVEM/CD160 complex inhibits the activation of CD4^{+} T lymphocytes. Understanding the interactions between cancer cells and immune system cells, using the example of HVEM/CD160 receptors, is essential to design therapeutic molecules (peptides or peptidomimetics) that will stimulate the body's immune response.

The subject of this doctoral dissertation was the structural studies of HVEM and CD160 proteins. Structural studies require large amounts of protein with a high degree of purity. Therefore, the first step of the research was to develop an efficient method for the expression and purification of the extracellular domains of HVEM$^{(39\text{-}199)}$ and CD160$^{(27\text{-}157)}$ responsible for complex formation.

HVEM$^{(39\text{-}199)}$ protein expression was performed in a bacterial expression system in two *Escherichia coli* strains: BL21(DE3) and SHuffle® T7. The protein was present in both soluble and insoluble fractions. Three different forms of HVEM$^{(39\text{-}199)}$ protein were overproduced in *Escherichia coli* cells: dimer, trimer and higher oligomer. In the case of recovery of the protein accumulated in the insoluble fraction, the ratio of obtained oligomeric forms differed depending on the refolding method used.

In the case of the CD160$^{(27\text{-}157)}$ protein, expression was carried out in three expression systems: bacterial, yeast and eukaryotic. Overproduction in the bacterial expression system, similarly to HVEM$^{(39\text{-}199)}$, was carried out in two *Escherichia coli* strains: BL21(DE3) and SHuffle® T7. The protein was completely accumulated in the insoluble fraction. In *Escherichia coli*, overproduction of the non-glycosylated monomer of the CD160$^{(27\text{-}157)}$ protein was possible, while glycosylated CD160$^{(27\text{-}157)}$ protein was obtained in yeast *Pichia pastoris* and in human embryonic kidney HEK293 cells. However, the obtained protein was not suitable for further studies. CD160$^{(27\text{-}157)}$ which was expressed in yeast, contained numerous sugar residues that were difficult to

completely cleave using enzymes. However, the amounts of protein overproduced in the eukaryotic expression system were too small to be used for crystallographic studies.

Subsequent structural investigations employed X-ray diffraction and small-angle X-ray scattering (SAXS).

Crystallization of both proteins was conducted using the vapor diffusion hanging drop method. Selected crystals of the HVEM$^{(39-199)}$ protein were soaked in gD7Nle peptide solution, an inhibitor of the formation of the HVEM/BTLA complex. The interaction of the peptide with the obtained protein was confirmed by affinity chromatography, electrochemical impedance spectroscopy and cyclic voltammetry. Screening for initial conditions of crystallization and their optimization allowed obtaining numerous crystals, which were subjected to diffraction studies using a synchrotron radiation source. Diffraction data were successfully collected for the HVEM$^{(39-199)}$ dimer and trimer purified from the soluble fraction.

SAXS experiments determined the radius of gyration (Rg) for both proteins: 3.03(2) nm for HVEM$^{(39-199)}$ and 2.48 nm for CD160$^{(27-157)}$. Regardless of concentration, the scattering curves suggested that CD160$^{(27-157)}$ monomer recovered from the insoluble fraction exhibited aggregation tendencies. This instability and potential oligomerization were further supported by dynamic light scattering results. Conversely, SAXS data confirmed that the HVEM$^{(39-199)}$ dimer purified from the soluble fraction was a globular protein. Additionally, ab initio modeling using the DAMMIF program generated a preliminary solution structure model for this dimer.