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Generation of an antibody toolbox to characterize hERG

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ABSTRACT

The human ether-a-go-go related gene (hERG) potassium channel plays a major role in the repolarization of the cardiac action potential. Inhibition of the hERG function by mutations or a wide variety of pharmaceutical compounds cause long QT syndrome and lead to potentially lethal arrhythmias. For detailed insights into the structural and biochemical background of hERG function and drug binding, the purification of recombinant protein is essential. Because the hERG channel is a challenging protein to purify, fast and easy techniques to evaluate different expression, solubilization and purification conditions are of primary importance. Here, we describe the generation of a set of 12 monoclonal antibodies against hERG. Beside their suitability in western blot, immunoprecipitation and immunostaining, these antibodies were used to establish a sandwich ELISA for the detection and relative quantification of hERG in different expression systems. Furthermore, a Fab fragment was used in fluorescence size exclusion chromatography to determine the oligomeric state of hERG after solubilization. These new tools can be used for a fast and efficient screening of expression, solubilization and purification conditions.

1. Introduction

The ether-a-go-go related gene encodes the conducting α-subunit of the human voltage-activated K^+ channel hERG [1]. Four subunits co-assemble to form the homotetrameric hERG channel, whose potassium permeable pore is formed by the interface of the four subunits [2]. Each subunit consists of six transmembrane helices with intracellular N- and C-termini [1]. The N-terminal cytosolic part is built up by a PAS (Peer-Arnt-Sim) domain [3] followed by 270 amino acids of unknown structure and function. The channel has six transmembrane helices (S1–S6) and a pore helix (P), with the voltage sensor formed by helices S1 to S4 and the pore domain comprising S5, P and S6 [1,2]. The C-terminal cytosolic part comprises a cyclic nucleotide binding domain (CNBD domain) [1], however a binding of cyclic nucleotides could not be shown so far. At the moment, only little is known about the spatial arrangement of the domains within the tetramer. Between the PAS and CNBD domains oligomeric associations were found, which were identified by mutations of surface exposed residues. The data suggest that the CNBD domain forms an inner ring directly beneath the transmembrane domains surrounded by an outer ring of the PAS domains [4,5].

hERG is expressed in the central nervous system [6,7], the human jejunum [8] and the endocrine system [9,10]. However, the most abundant expression is found in the heart, where it is responsible for the rapid component of the delayed rectifier K^+ current (I_{Kr}) [11]. I_{Kr} plays a key role in maintaining the electrical stability of the heart [12]. Alterations in the functional properties by genetic mutations or by administration of drugs of different class and structure suppress I_{Kr}-function and are linked to congenital and acquired long QT syndrome [13]. With recent emphasis in efficient drug development to decrease costs and attrition rates, it is highly desirable to evaluate compounds for their potential to trigger long QT syndrome as early as possible and ideally during early lead optimization [14].

Beside these innate hERG expressing tissues, a growing number of hERG expressing cancer cell lines have been identified in recent years. These studies suggest that hERG channel expression is associated with aggressive tumors and may be involved in mediating invasion. Therefore, hERG channel expression was discussed as a diagnostic or even therapeutic target (reviewed in [15,16]).

Regarding the fundamental roles of hERG in the congenital and acquired long QT syndrome and several cancer forms, it is crucial

Abbreviations: hERG, human ether-a-go-go related protein; mAB, monoclonal antibody; FSEC, fluorescence size exclusion; RPC, reversed phase chromatography; Fab, fragment antigen-binding.

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to obtain detailed biophysical information about the residues in the hERG channel that are involved in drug binding. Thus the production of recombinant protein is essential. Because the hERG protein is a relative unstable oligomeric mammalian membrane protein, overexpression and purification is very difficult. Therefore establishing reproducible, fast and sensitive assays to judge quality and function of the protein *in vivo* during expression screens and *in vitro* during purification is of prime importance. Besides established tools like radioactive binding assay, patch clamp measurements or western blot we aimed for tools which allow an unprecedented characterization of hERG in a solubilized state.

Here we describe the generation of monoclonal antibodies raised against purified hERG as tools for better characterization and purification. With these antibodies we were able to set up a fast, sensitive and cost-effective ELISA based assay for the relative quantification of hERG within cell lysates, membrane preparations or solubilized membrane fractions. Fab fragments of antibodies labeled with a fluorescence marker have been used to probe the oligomerization state of untagged hERG by fluorescence size exclusion chromatography (FSEC) at different purification steps.

2. Materials and methods

2.1. Cloning of full-length hERG

The synthetic gene of Kv11.1 (hERG1) was ordered from GenScript, USA and cloned into the pFastBac vector (Invitrogen) with the restriction sites *BamHI* and *NotI* (New England Biolabs, USA) either with a C-terminal Strep- and 8xHis tag for purification (IBA GmbH, Germany) or with a C-terminal GFP and 8xHis tag, separated with a five amino acid linker.

2.2. Protein expression in Sf9 insect cells

Recombinant baculovirus particles were produced according to the Invitrogen manual. To express hERG-Strep-His, 2–2.5 $\times$ 10^6 -cells/ml Sf9 cells were infected with 1.5% (v/v) recombinant baculovirus and cultured in fully-instrumented stirred tank bioreactors. Infected cells were harvested 48 h post-infection, washed once with ice-cold PBS and frozen.

2.3. Purification of hERG-strep-8xHis

Cells were lysed with a Basic Z cell disrupter (Constant Systems Ltd., United Kingdom) in (in mM) 50 sodium phosphate at pH 7.5, 300 NaCl, 20 KCl, 10 $MgCl_2$, 10% (v/v) glycerol, 2 dithiothreitol (DTT), 1 tablet/50 ml complete EDTA-free, 2 μg/ml pepstatin, 5 μg/ml E64, 40 μg/ml phosphoramidon, 5 diisopropyl fluorophosphate, 40 μg/ml DNaseI and RNase. After centrifugation at 5500*g* for 10 min, the supernatant was centrifuged at 120,000*g* for 60 min. Membrane proteins were solubilized at 10 mg/ml in (in mM) 50 sodium phosphate pH 7.5, 300 NaCl, 10 KCl, 10% (v/v) glycerol, 1 DTT and 4 tablets/l complete EDTA-free using 1.5% (w/v) Foscholine-12 (Fos-12) (Anatrace, USA). After centrifugation at 95,000*g* for 40 min, the supernatant was adjusted to 10 mM imidazole and applied to Ni–NTA agarose (Qiagen, Germany) column equilibrated with lysis buffer containing 0.15% (w/v) Fos-12 and 10 mM imidazole (equilibration buffer) on an Aekta-FPLC and washed with 20 mM imidazole, then 40 mM imidazole and eluted with 250 mM imidazole. Protein fractions were pooled and concentrated with an Amicon Ultra-4 Ultracell 50 K centrifugal filter (Millipore, USA) and loaded on a Superose 6 column (GE Healthcare, USA) equilibrated with (in mM) 50 Tris/HCl at pH 7.5, 295 NaCl, 5 KCl, 10% (v/v) glycerol and 0.15% (w/v) Fos-12. hERG-containing peak fractions were identified by reversed phase chromatography (RPC) as described below, pooled and concentrated. A fraction of the concentrated pool was loaded on a TSK G4000SW column (Tosoh Bioscience, Japan) equilibrated with the same buffer and peak fractions were analyzed with RPC.

2.4. RPC–HPLC methodology for hERG analytics

RPC separations were performed on an Agilent 1200 Series HPLC with a C8 Poroshell 300SB 5 μm column with solvents A (water plus 0.1% TFA) and B (100% acetonitrile with 0.08% TFA). Chromatographic conditions were: 6 s isocratic A + 2.5% B, 42 s gradient to 10% solvent B, 102 s gradient to 65% B, 18 s gradient to 95% B and 6 s at 95% B. Fractions were collected and hERG retention time was identified with western blot analysis.

2.5. Generation of monoclonal antibodies against hERG

Twelve in house antibodies were raised in Naval Medical Research Institute (NMRI) mice injected intraperitoneally with 20 μg of purified hERG-Strep-8xHis, immunecomplexed with the monoclonal antibody His-6/9 (Hoffmann-La Roche Ltd., Switzerland; in-house) and emulsified in aluminum hydroxide gel (Alhydrogel-2%, Brenntag Biosector, Germany) containing CpG oligodeoxynuclotides (CpG-ODN) according to [17]. The rest of the antibody production and selection is described in the Supplemental information.

2.6. Immunofluorescence staining

All steps were performed at room temperature. CHO-Cre-Lox_hERG [18] cells were washed in PBS, resuspended, fixed and permeabilized by incubation with 0.1% (v/v) Triton X-100 in PBS for 10 min. After washing twice, cells were incubated in 0.1 mg/ml $NaBH_4$ in PBS for 10 min, washed and blocked by incubation in 3% (w/v) BSA in PBS for 1 h. Immunostaining was performed by incubation with an appropriate mAB dilution in blocking solution for 1 h. After three washes, 5 μg/ml F(ab′)2 fragment-Cy3 goat anti-mouse IgG antibodies (Jackson Immunoresearch, USA) diluted in blocking solution was added and incubated for 1 h. After three washes, cells were mounted with mounting medium containing DAPI (ProLong® Gold antifade reagent with DAPI, Invitrogen, USA).

2.7. hERG ELISA assay

All steps were performed in a humid incubator. Microtiter plates (MaxiSorb, NuncTM, Denmark) were coated with purified anti-hERG antibody 4/32 (5 μg/ml diluted in PBS), washed twice with PBST and blocked for 1 h at 37 °C with (in mM) 50 Tris/HCl pH 7.4, 139 NaCl, 5 EDTA, 0.05% (v/v) Nonidet P40 substitute, 0.25% (w/v) gelatine and 1% (w/v) bovine serum albumin (BSA). After washing twice with PBST, the wells were incubated for 1 h at 37 °C with cell lysates of CHO-hERG, CHO-K1, hERG expressing HEK293 cells (HEK-hERG), HEK293 wildtype cells, hERG-Strep-8xHis expressing Sf9 cells, Sf9 wildtype cells or HL60 cells in NP40 buffer and cell lysates were diluted in blocking buffer. After four washes in PBST, mouse anti-hERG antibody 4/74-HRP (0.5 μg/ml diluted in blocking buffer) was added. The anti-hERG-HRP was detected with 3,3′,5,5′-tetramethyl-benzidine (TMB) and the absorbance at 450 nm was measured.

To compare the detection antibody of this work with the commercially available mAB A12 (Enzo Life Sciences, USA), we performed an indirect ELISA by coupling dilutions of the cell lysate directly onto the microtiter plates. After blocking, the primary antibodies were used (0.5 μg/ml) and detected with goat anti-mouse-HRP IgG (Jackson Immunoresearch, USA) (0.08 μg/ml). All other steps were identical with the sandwich ELISA protocol.

2.8. FSEC with labeled Fab

The generation and labeling of the Fab fragment is described in the Supplemental material. HEK293 and HEK-hERG membranes were prepared as described under purification. The membranes were resuspended with cell lysis buffer in a Dounce homogenizer, adjusted to OD_{280} of 10. They were solubilized for 45 min at 4 °C by adding DDM to 1% (w/v) and 1 mg Fab 4/21-Alexa488. After centrifugation at 60,000g for 20 min 500 ml was loaded onto a Superose 6 column (10/300 GE) equilibrated in (in mM) 50 Tris/HCl pH7.5, 295 NaCl, 5 KCl, 10% glycerol and 0.05% DDM and connected to an Aekta purifier in line with an Agilent 1100 FLD for recording fluorescence (excitation 494 nm; emission 520 nm, PMT gain 16).

3. Results

3.1. Expression and purification of hERG-strep-8xHis

A critical step for the generation of monoclonal antibodies against hERG was that purified hERG instead of synthetic peptides was used for immunizing mice. The hERG channel was already expressed in mammalian cell lines such as HEK293 and CHO-K1 [19–21]. However, up to date hERG was never reported in the literature to be overexpressed in large amounts for purification purposes. In our study we used the Sf9/Baculovirus system to overexpress the full-length hERG. Using a C-terminal GFP-tagged variant of full-length hERG (hERG-GFP-His), the localization of the channel in the cell was documented via confocal laser scanning microscopy (Fig. 1A). This clearly indicated that the protein was integrated into the membrane and transported to the cell surface. Additionally, whole-cell patch-clamp of Sf9 cells showed hERG specific currents, which could be blocked by the hERG specific inhibitor astemizole (data not shown).

We tested more than 70 different detergents in purification trials, but only Foscholine-9 to -16, Cyclofos-6,-7 and TDAO did solubilize hERG-Strep His. The size-exclusion chromatogram of hERG after IMAC in Foscholine-12 shows a broad peak with several shoulders (Fig. S1). We analyzed each fraction with reversed phase chromatography for the relative hERG content and plotted the corresponding hERG peak integral (Fig. S1, dotted line). A rechromatographed aliquot of the pooled size-exclusion chromatography (SEC) fractions showed one major peak at a molecular weight of about 500 kDa, determined with molecular weight standards (Fig. 1B). But multi-angle-light-scattering (MALS) revealed a molecular weight of about 120 kDa for the protein and 190 kDa for the protein/detergent conjugate. CD measurements indicated a predominantly α-helical secondary structure composition. This suggests that purified hERG adopts a folded, but monomeric, non-functional state. Purification using other Foscholines or TDAO also resulted in monomeric, non-functional hERG.

3.2. Generation and purification of mABs

Monomeric purified hERG was used for the antibody production. After selection against active, membrane embedded CHO-hERG in an immunofluorescence assay, we were able to isolate 12 clones producing monoclonal antibodies against hERG. To our knowledge, these are the sole monoclonal antibodies raised against purified hERG. The immunoglobulin isotype analysis revealed that the mABs belong to the subclass IgG2a, except for mAB 4/69 and mAB 4/74, which belong to IgG2b.

3.3. Western blot analysis

These 12 antibodies were able to detect hERG in western blots of CHO-hERG cell lysates, indicating that a linear epitope is sufficient for binding (Fig. S2A). The mABs 4/14, 4/114 and, to a minor degree, 4/66 detected also one additional band of a protein from hERG-negative CHO-K1 cell lysates. As negative control an in house mouse monoclonal antibody against a His tag was used.

The applicability of the antibodies for immunoprecipitation was tested with the same cell lysates. In all cases mAB 4/27 coupled to horseradish peroxidase was used for detection of the immunoprecipitated hERG channel. Western blot analysis of the immunoprecipitates resulted in specific strong bands at the apparent molecular weight of hERG (Fig. S2B).

3.4. Epitope localization

To analyze the epitopes detected by the new antibodies, three different truncations were prepared: $hERG_{1-870}$-His, $hERG_{1-362}$-GFP-His and $hERG_{1-130}$-GFP-His (see Supplementary material). Western blot analysis using these constructs revealed the two epitopes 130–362 and 870–1159 in the cytoplasmic regions (Fig. 2C). The localization of the epitopes for the antibodies 4/14 and 4/84 was less conclusive.

3.5. Immunofluorescence staining of hERG in CHO cells

As the antibodies detect native linear epitopes within the cytoplasmic parts of hERG (Fig. 2C), they can be useful for the detection of endogenous hERG by immunofluorescence staining of CHO-hERG cells. All antibodies were unable to detect hERG in non-permeabilized cells, due to the recognition of intracellular epitopes, and are therefore not suitable for FACS.

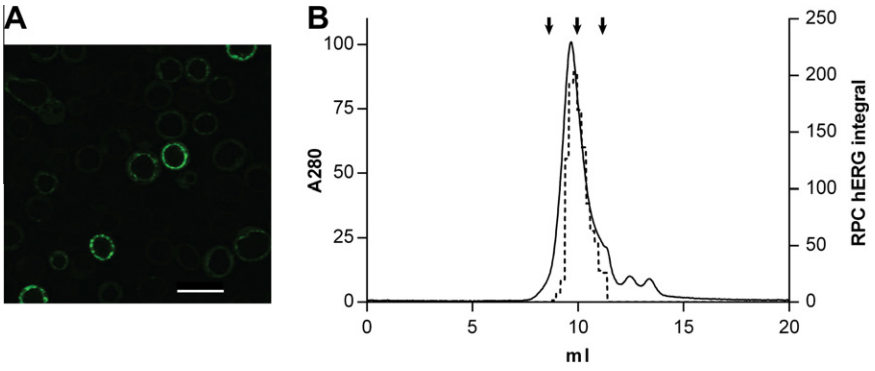

Fig. 1. Expression and purification of full-length hERG-Strep-His. (A) Confocal microscopy of Sf9 cells expressing hERG-GFP. The scale bar corresponds to 30 μm. (B) Size exclusion chromatogram of purified full-length hERG reinjected on a TSK G4000SW column. The corresponding RPC derived hERG peak integrals are plotted (histogram). Retention volumes of thyroglobulin (669 kDa), ferritin (440 kDa) and albumin (67 kDa) are indicated.

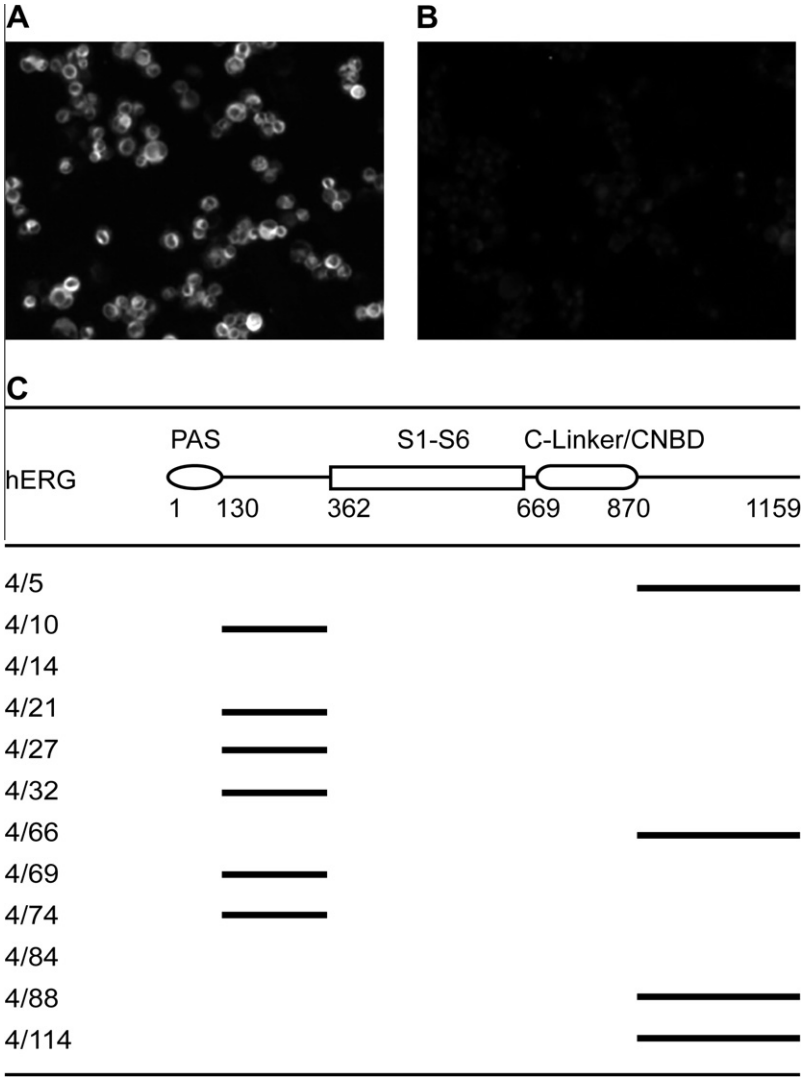

Fig. 2. Immunofluorescence staining of CHO cells. (A) CHO-hERG and (B) CHOK1 cells were incubated with the mAB 4/74 coupled to Alexa Fluor 488 and photographed under a fluorescence microscope. (C) Epitope localization. The antibody clones were tested for their capability to detect the different hERG-truncations on western blots. The binding areas are depicted as black bars.

In contrast the permeabilized CHO-hERG cells exhibited a strong fluorescence mainly localized at the cell surface (Fig. 2A). Whereas the permeabilized hERG negative CHOK1 cell line showed only a weak background signal (Fig. 2B).

3.6. hERG sandwich ELISA with mABs

The major reason for the generation of new monoclonal antibodies against hERG was to establish a sensitive sandwich ELISA for the detection and relative quantification of hERG in cell lysates. The ELISA was established using CHO-hERG cells and CHO-K1 cells as a negative control. Each of the antibodies was utilized as either the capture or, in the horseradish peroxidase coupled form, the detection antibody in order to determine the optimal combination. Although both antibodies bound to antigens located between the PAS and the transmembrane part, the epitopes were non-overlapping. This sandwich ELISA was highly specific, being capable to detect hERG in a dilution of 2000 cells/ml whereas the corresponding hERG-negative CHO cell line showed almost no background (Fig. 3A).

In addition we compared the antibody mAB A12 from Enzo Life Sciences (USA) generated against a peptide of the extracellular loop S5-P with the detection antibody mAB 4/74 in an indirect ELISA using the unconjugated form of mAB 4/74 and mAB A12 and a peroxidase conjugated goat anti-mouse IgG as secondary antibody. The mAB 4/74 exhibits both, a two to threefold higher signal and a higher sensitivity, with similar background levels. As a possible application for this ELISA we analyzed the relative amounts of hERG expressed in different cells (Fig. 3C). The strain with the highest expression was the PrecisION HEK-hERG (Millipore, USA) followed by the CHO-hERG cell line. The expression of hERG in transfected Sf9 cells, which was used for the purification of hERG in this work, is comparable to the expression in CHO cells. Using the ELISA, we could also detect and quantify the relative amount of hERG in the human promyelocytic leukemia cell line HL60 [22], which was shown to constitutively express the hERG gene [23]. In addition, it was possible to detect the binding of solubilized hERG to mAB 4/32, immobilized on a sensor, with bio-layer interferometry (ForteBio, data not shown) offering applications as described by Myszka et al. for GPCRs [24].

3.7. Fab fragment labeling and FSEC

The oligomeric state of hERG could be estimated by fluorescence size exclusion chromatography (FSEC) in solubilized membrane fractions using fluorescently labeled antibodies. To

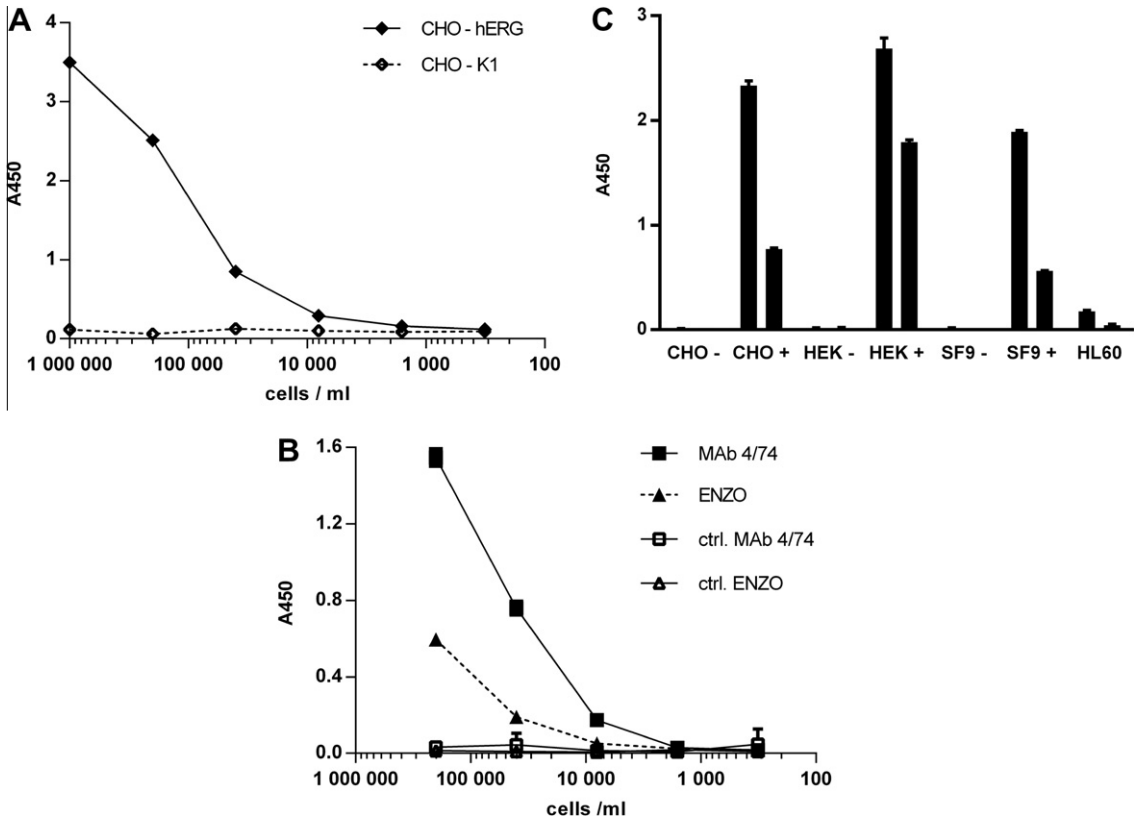

Fig. 3. ELISA for the detection of hERG in cell lysates. (A) Sandwich ELISA of CHO-hERG and CHOK1 lysates. mAB 4/32 was used for coating and mAB 4/74-HRP for detection. (B) Comparison of mAB 4/74 and mAB A12 (Enzo Life Sciences) as primary antibody in an indirect ELISA. PrecisION HEK-hERG and HEK293 cell lysates were bound directly to microtiter plates and probed with mAB 4/74 and mAB A12, respectively and detected with goat anti-mouse-HRP. (C) Sandwich ELISA of different cell lines at 1 mio/ml and 0.2 mio/ml cells either expressing (+) or not expressing (−) hERG.

circumvent possible multimerization difficulties of an antibody, we generated a Fab fragment of the mAB 4/21, purified and labeled it with Alexa Fluor 488 and complexed it with DDM solubilized membranes of HEK-hERG cells for subsequent FSEC. As a control HEK wildtype membranes were loaded (Fig. 4A). The fluorescence signal shows a large peak originating from the Fab. In contrast, a distinct fluorescence signal could be observed, when the labeled Fab 4/21 was incubated with DDM solubilized membranes of hERG PrecisION HEK cells (Millipore, USA) (Fig. 4B). From previous purifications we knew that the retention volume of about 15.5 ml on a Superose 6 column corresponded to monomeric hERG (Fig. S1A). Furthermore, possible oligomeric species of hERG could be detected.

4. Discussion

The antibodies described here bind to native linear surface epitopes of the cytoplasmic part of hERG recognizing it in a functional state in the membrane. We did not find mABs against the CNBD and transmembrane domain, likely because mABs were selected with active hERG in CHO cells and current hERG models have an inner ring of CNBD domains buried by an outer ring of PAS domains [4,5]. mABs against the PAS domain were also not selected, since the sequence identity between hERG and mERG is extremely high (96%). Rather most antibody epitopes cluster in the region 130–320, which correlates with the prediction of a high degree of disorder and might explain why all antibodies recognize linear epitopes. This region is only present in hERG isoform 1a, therefore our antibodies allow to discriminate between hERG1a and hERG1b [25]. The ELISA with the newly produced antibodies was highly sensitive and it could verify the constitutive expression of hERG in the HL60

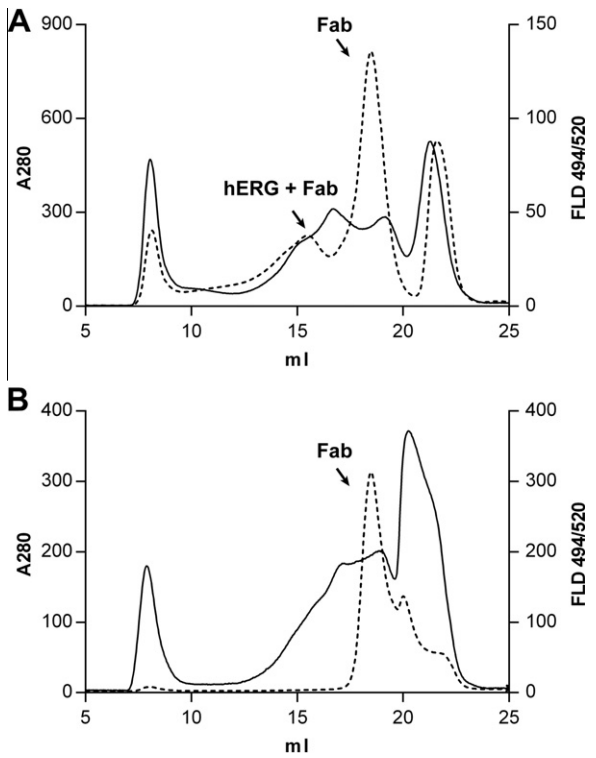

Fig. 4. FSEC of solubilized, Fab 4/21 labeled HEK membrane fractions. (A) FSEC of DDM solubilized membranes of HEK293 control cells and HEK-hERG (Millipore) cells (B). The membrane fraction was solubilized with DDM, incubated with fluorescently labeled Fab 4/21 and loaded on a Superose 6 column. The UV absorbance and fluorescence (dashed line) are depicted.

cell line [23]. This ELISA setup could be used to detect and quantify hERG expression levels in various cancer cell lines.

In conclusion, the difficulties which arose during the attempt of purifying hERG demonstrate the importance of having high-throughput adaptable tools for the expression and purification screening. Studies with GPCRs have exemplified this need to find the conditions and mutations which promote protein stability [26,27]. The FSEC method with labeled Fab could be used for a fast assay of the oligomeric state of hERG. Furthermore, the hERG mAbs will allow using other methods like Biacore or immune affinity chromatography.

Our antibody based methods for sensitive and quantifiable detection provide the possibility to optimize the expression and purification of hERG for *in vitro* functional and structural analysis and to pursue protein engineering studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.089.

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