Role of calcium ion and anionic phospholipids on the ubiquitin aggregation process

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INTRODUCTION

Ubiquitin (Ub) is a protein found in all eukaryotic cells, with a well-conserved primary sequence across species. It is composed of 76 amino acids and has a molecular weight of 8565 Da. Human ubiquitin (hUb) has been identified in various cellular locations such as the nucleus, cytoplasm, and cell membrane, although its main role is intracellular (Vijay-Kumar S et al., 1987). Ubiquitin participates in numerous biological processes, including the cell cycle, lymphocyte differentiation, and ATP-dependent protein degradation. The Ubiquitin-Proteasome system (UPS) is the primary mechanism for degrading misfolded or damaged proteins. This process involves several steps: activation of Ub by the ubiquitin-activating enzyme (E1) in an energy-dependent manner, transfer of Ub to the ubiquitin-conjugating enzyme (E2), and the subsequent transfer of Ub to the target substrate catalyzed by the ligase enzyme (E3).

The failure of the UPS in dealing with misfolded proteins may play a role in certain neurodegenerative diseases. Research has indicated a link between protein misfolding, aggregation, UPS and neurodegenerative disorders. In some cases, proteins prone to forming aggregates may become ubiquitylated, leading to the formation of ubiquitin-positive aggregates. Alternatively, the accumulation of ubiquitin-positive inclusions may be caused by dysfunction of the proteolysis system (Morimoto D et al., 2016). Ubiquitin-positive protein aggregates have been identified in senile plaques and Lewy bodies, which are the hallmarks of Alzheimer's and Parkinson's disorders, respectively (Arnesano F. et al., 2009). Several studies have shown that the stability of Ub is influenced by binding to certain metal ions or membrane lipids. Copper (Cu^{2+}) and zinc (Zn^{2+}) can bind to Ub, promoting its aggregation, as well as anionic lipids acting as molecular chaperones.

In this investigation, we studied the effects of calcium ions (Ca^{2+}) and anionic phospholipids on the aggregation process of human ubiquitin using various techniques.

MATERIALS and METHODS

Gel electrophoresis. The aggregation state of hUb was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4-20% gradient precast TGX gels (Biorad). For this assay, three samples were prepared. The first sample contained hUb alone (10 µM), the second hUb and phosphatidylserine (PS) at the concentration of 100 µM and the third hUb, PS and Ca^{2+} at the concentration of 100 µM. Samples were pre-incubated for 96 hours at room temperature then were mixed with Laemmli buffer and boiled for 5min at 95 °C. The gels were run at 150 V in TGS running buffer and stained with silver nitrate.

Single-channel measurement. Channel activities were recorded in planar lipid membranes (PLMs) made up of Palmitoyl-oleoyl-phosphatidylserine (POPS, Avanti Polar Lipid) in 1% n-decane (Sigma), as detailed in previous descriptions (Meleleo D et al., 2023). The method used in the single-channel measurements remains consistent with our earlier studies (Meleleo D, 2021). To evaluate the channel activity of hUb in the absence and in the presence of Ca^{2+}, three experimental sets were set up. The final hUb concentration used in the three sets was 1 µM. In the first experimental set, the channel activity of hUb alone was monitored, while in the second and third sets, it was monitored in the presence of 100 and 300 µM of $CaCl_2$, respectively. To determine conductance, a histogram of the conductance amplitude distribution for each experimental set was constructed and fitted by a Gaussian distribution function. Results are expressed as central conductance ± standard error (Λ_c ± SE). To determine the frequency (number of channels in 60 s), any detection of channel events was counted as successful. Results are expressed as frequency ± standard deviation (F ± SD).

Relaxometry measurements. For relaxometry measurements, large unilamellar vesicles (LUVs) were prepared by following the thin lipidic film method. Briefly, Dipalmitoylphosphatidylcholine (DPPC) and Dipalmitoylphosphatidylserine (DPPS), at molar ratio DPPC:DPPS=65:35,w/w, were dissolved in chloroform (20 mg/mL). The solution was slowly evaporated to form a thin film. The film was then hydrated at 55°C with an aqueous solution containing 200 mM of Gd-HPDO3A (300 mOsm/L). The final suspension of LUVs was purified and the vesicles were investigated by dynamic light scattering (Zetasizer NanoZS, Malvern, UK) to assess the mean hydrodynamic size and the polydispersity of the system. The T_1 of the liposome suspensions was measured using a Stelar Spinmaster spectrometer (Stelar, Italy) via the standard inversion recovery (180°-τ-90°C) pulse sequence. Since $T_1 = 1/ R_{1obs}$ and $R_{1obs} = R_{dia} + [Gd^{3+}]\times R_{1\rho}$, $R_{1\rho}$ was used to compare each sample, avoiding differences due to gadolinium concentration. An increase in water permeability corresponds to an increase in the $R_{1\rho}$ value. The protein incubations and kinetic studies were conducted with a protein:lipid ratio of 1:80, upon addition of 10 mM EDTA to chelate traces of divalent metal ions present in the solutions.

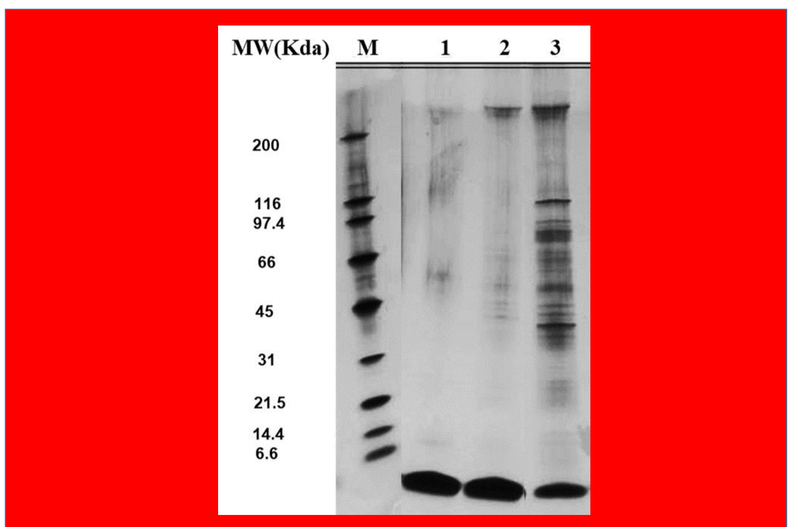

Fig.1 Aggregation states of hUb alone (line 1), in presence of PS (line 2) and PS and Ca^{2+} (line 3) analyzed with SDS-PAGE. Each sample contains 10 µM hUb with 100 µM PS (sample 2) and 100 µM + 100 µM Ca^{2+} (sample 3).

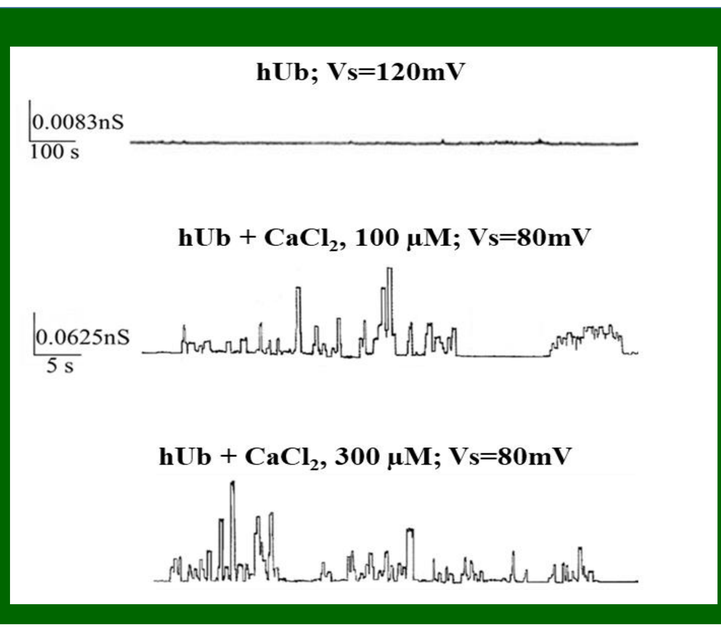

Fig. 2 Example of chart recording of hUb in POPS PLM. Experimental conditions: KCl 1 M (pH 7), hUb final concentration 1µM, T = 23±1°C.

$[CaCl_2]_{cis/trans}$ (µM)	Λ_c±SE (nS)	F±SD
0	--	--
100	0.026±0.002	1.73±0.20
300	0.025±0.001	2.79±0.18

Table 1 Mean conductance (Λ_c±SE) and frequency (F±SD) of hUb channel in absence and in presence of different $CaCl_2$ concentrations. Experimental conditions: KCl 1 M (pH 7), hUb final concentration 1µM, T = 23±1°C.

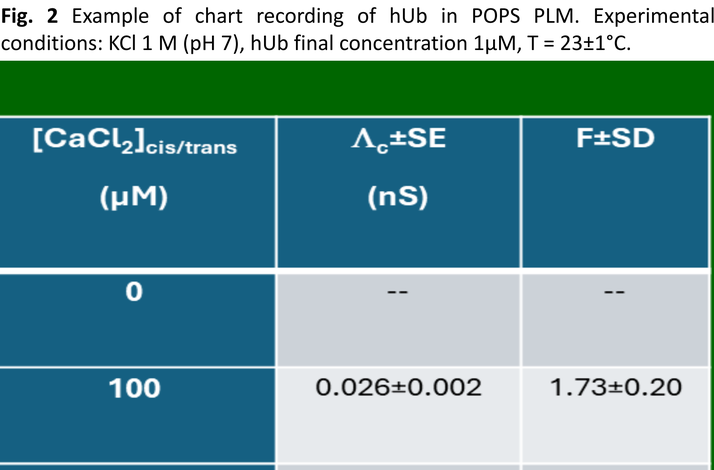

Fig. 3 Change in water permeability of liposomes incubated with hUb in the absence and presence of Ca^{2+}. $R_{1\rho}$ is directly related to the water permeability of liposomes. Experimental conditions: Liposomes were made up of DPPC:DPPS= 65:35, w:w; Phospholipids concentration 20 mg/mL; Molar ratio hUb: Phospholipids=1:80; Ca^{2+} concentration 1mM; EDTA concentration 10 mM.

RESULTS

Gel electrophoresis. The aggregation state of hUb in the three different experimental conditions was shown in Fig. 1. It is important to note that, only in the presence of Ca^{2+}, hUb shows two bands between 45 and 66 KDa presumably corresponding to pentamers and octamers and one band at the apparent molecular weight (MW) of 97.4 kDa. The results show that: - hUb alone and in the presence of PS is predominantly in monomer form; - hUb incubated with PS and Ca^{2+}, for 96 hours, forms oligomers indicating that the divalent ion promotes protein aggregation.

Single-channel measurement. Oligomers and soluble aggregates, unlike fibrils and monomers, show the ability to permeabilize the membrane through the formation of conductive units or with a detergent-like mechanism (Nguyen PH et al 2021; Zhao J et al 2012). To evaluate the hUb ability to interact with membrane lipids we carried out experiments using the electrophysiological technique of single-channel current measurements. For the first experimental set, five different experiments were conducted in each of which, after the addition of hUb stock solution at the applied voltage of 80 mV, no variations in the biophysical parameters of the membrane were observed for long periods and under the application of voltages as high as ±120 mV. In the second and third series of experiments, the effect of 100/300 µM $CaCl_2$ on hUb ability to incorporate into PLM and form ion channel was evaluated. $CaCl_2$ 20/60 µL of stock solution was added at 80 mV. After approximately 5.30/1 hours from Ca^{2+} addition we observed sudden current jumps from the baseline membrane current characterized by different levels of conductance and compatible with channel-type openings and closures (Fig. 2). Channel activity alternated with long periods of quiescence and disappeared completely after approximately 90 min from the manifestation of the first channel, even under the application of voltages as high as ±100 and ±120 mV. Conductance (Λ_c±SE) and frequency (F±SD) values of the hUb ion channel in the absence and presence of $CaCl_2$ are reported in Table 1. The results obtained from the single channel experiments show that: - hUb is not able to incorporate into POPS PLMs and to form ion channels; - Ca^{2+} ion promotes the incorporation of hUb into the negatively charged membrane and the formation of the ion channel indicating that, in solution, Ca^{2+} promotes the aggregation of hU molecules to oligomers prone to incorporate into the membrane and to form conductive units.

Relaxometry measurements. The ability of hUb, in the absence and the presence of Ca^{2+}, to interact with bilayer lipids was evaluated by studying the permeabilization of liposomal vesicles using paramagnetic probes. It is known that the interaction of some proteins with the membrane is driven by electrostatic interactions, for this reason, we have carried out one series of experiments in which the liposomes were made up of DPPC:DPPS. Fig. 3 shows that the $R_{1\rho}$ values obtained by incubating the liposomal vesicles with hUb in the presence of Ca^{2+} are higher than those incubated with hUb alone, indicating that the permeability of the liposomes to water molecules increases in the presence of the ion. These results suggest that hUb in the presence of Ca^{2+}, interacting with the lipids, can permeabilize the liposome membrane, differently from the protein alone.

CONCLUSION

The results of this preliminary study show that: - membrane permeabilization of DPPC:DPPS= 65:35 liposomes is significantly increased when lysosomal vesicles are incubated with hUb in the presence of Ca^{2+}; - calcium ions promote the formation of hUb oligomers, probably through chelation of the amino acid Glu at position 16 of the primary sequence of the protein; - hUb oligomers are prone to interact with POPS PLMs forming conductive units. These results strengthen the hypothesis of the link between hUb protein malfunction and neurodegenerative diseases suggesting a possible mechanism of action promoted by Ca^{2+} ions and membrane lipids.