The role of IRSp53 and RAGE proteins in the formation of membrane nanotubes

PhD Thesis

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1. Introduction

Communication between cells can occur through the transfer of information and materials via membrane nanotubes. Membrane nanotubes are capable of establishing long-range physical connections between distant cells, enabling the transport of various cellular components and signaling molecules. Their formation is based on complex molecular mechanisms in which actin filaments, as well as proteins that generate membrane curvature and regulate actin polymerization, play a central role.

The aim of our work was to explore the mechanism of membrane nanotube formation, with particular emphasis on the role of the IRSp53 protein (Insulin receptor substrate protein 53 kDa), which is capable of inducing plasma membrane curvature and promoting nanotube-like structures. For our investigations, we used two cell lines that form nanotubes via distinct mechanisms. The effects of IRSp53 and the I-BAR (inverse Bin-Amphiphysin-Rvs) domain on cellular and nanotube morphology were examined using confocal microscopy and SIM (Structured Illumination Microscopy). In addition, various in vitro techniques (e.g., TIRFM (Total Internal Reflection Fluorescence Microscopy) and a pyrene–actin polymerization assay) were employed to measure the effects of the full-length protein and its domain on actin polymerization.

In the second part of the research, we developed a system that enables the regulation of nanotube formation based on the activation and inhibition of the RAGE receptor (Receptor for Advanced Glycation End Products). Inhibition was achieved using the soluble form of the receptor, the sRAGE (soluble Receptor for Advanced Glycation End Products) protein, which can bind glycated proteins in the extracellular space. Receptor activation was carried out using ribose-glycated bovine serum albumin (RBSA). The sRAGE protein was purified from porcine lung using multiple chromatographic procedures and subsequently identified by HPLC-MS/MS (High-Performance Liquid Chromatography Tandem Mass Spectrometry). Protein–protein interactions were also verified by native gel electrophoresis. Finally, the effects of glycated protein and sRAGE on nanotube formation were investigated in the HEK 293 (Human Embryonic Kidney 293) cell line.

Based on our results, we concluded that both the I-BAR domain and the IRSp53 influence actin polymerization and facilitate the formation and elongation of membrane nanotubes by promoting actin nucleation. We successfully produced sRAGE of optimal purity and its ligand,

ribose-glycated bovine serum albumin (RBSA), which enabled us to regulate nanotube formation.

1.1. Membrane nanotubes

Membrane nanotubes (hereafter abbreviated as NTs, following the English term “nanotube”) are filopodium-like cellular protrusions that provide physical connections between distant cells. Based on studies performed in vitro cell cultures, filopodia that are morphologically similar to nanotubes adhere to the surface of the coverslip or culture vessel, whereas NTs float freely in the medium.

In vitro experiments indicate that NTs participate in various transport processes. Among others, they are involved in the intercellular transfer of calcium ions, lipids, numerous proteins (including misfolded proteins such as amyloid β, tau, and prions), nucleic acids, and immune costimulatory molecules, and they are even capable of transporting large cellular organelles. NTs contain cytoskeletal filaments (e.g., actin and microtubules). These structures not only form the scaffold of NTs but, through the action of motor proteins, can also mediate certain transport processes and − similarly to filopodia − are indispensable for NT formation.

Despite increasing evidence for the physiological functions of NTs, the molecular mechanism underlying NT growth is not yet fully understood. NT formation can be influenced by environmental factors (e.g., reduced oxygen levels, high glucose concentration, serum deprivation, UV radiation), certain bacterial and viral infections, as well as specific proteins.

To date, two main strategies have been described by which different cell types can form NTs.

1. One mode of NT formation is based on an actin-driven protrusion mechanism. In this case, a filopodium-like protrusion develops on the surface of one or both cells, and following elongation of these protrusions, the cells establish contact with each other.
2. Another possibility is the cell dislodgement-based mechanism, in which two cells that are initially in physical contact move away from each other in opposite directions while remaining connected via NTs (a process characteristic of motile cells, such as T and B lymphocytes).

Certain nanotubes are open at both ends, meaning that the cytoplasm of the two connected cells is in direct continuity. Such NTs have been observed, among others, in B lymphocytes, macrophages, and between PC12 cells. Open-ended tubes can be classified into three distinct subgroups as follows.

1.) *Thin nanotubes* derive their name from the fact that their diameter does not exceed 0.7 μm. They contain F-actin (filamentous actin) but lack microtubules. Membrane-bound bacteria and other particles can "surf" along the surface of these thin tubes through continuous lateral membrane movement. This flow represents active transport and does not require microtubules.

2.) *Thick nanotubes* contain both F-actin and microtubules and have a diameter exceeding 0.7 μm. Their surface does not exhibit lateral membrane movement as observed in thin nanotubes; however, active transport of various organelles (e.g., endosomes, lysosomes, mitochondria) and vesicles can be detected within the lumen of the tube. Vesicle movement typically occurs bidirectionally, suggesting that multiple independent "tracks" may be established within the same tube. The relative contribution of actin and microtubules to intercellular active transport is likely cell line-dependent.

3.) Structures that appear as a single tube under fluorescence microscopy may in some cases consist of multiple individual membrane nanotubes (iTNT – *individual Tunneling NanoTube*). iTNTs run in bundles, parallel to one another and sometimes twisting around each other. This configuration enhances stability and flexibility, allowing such NTs to withstand forces generated by cellular movement. Vesicles and other organelles can be observed within iTNTs moving along actin filaments, indicating active transport through the tubes. While most iTNTs possess open ends, closed terminal structures may also be observed in certain cases, suggesting variability in their mode of cellular connection.

1.2. Actin

Actin is essential for the formation of NTs and also plays an important role in their maintenance. The actin monomer is structurally divided into two major domains (α and β domains) and consists of four subdomains. Its monomeric form is referred to as G-actin (globular actin), whereas its polymerized form is known as F-actin (filamentous actin).

The first step of actin polymerization is nucleation, during which a stable oligomer − typically composed of three to four G-actin molecules − is formed with the assistance of actin-nucleating proteins. Additional G-actin units accumulate at both ends of the oligomer, initiating the second phase, elongation, in which the filament continues to grow, and F-actin is formed. During polymerization, a plus end (barbed end) and a minus end (pointed end) are distinguished. These terms refer to the rates of polymer growth and depolymerization. When sufficient G-actin is available, a dynamic equilibrium (steady state) can be established, in which the rates of subunit

association and dissociation are nearly identical at the two ends of the filament, resulting in a continuously renewing polymer chain. This equilibrium is influenced by the concentration of free G-actin and ATP, as incorporation of actin monomers into the filament occurs through ATP binding, which is subsequently hydrolysed within F-actin.

1.3. IRSp53 protein and its I-BAR domain

Insulin receptor tyrosine kinase substrate 53 (IRSp53) can interact simultaneously with the plasma membrane and the actin cytoskeleton. Through its inverse Bin-Amphiphysin-Rvs (I-BAR) domain, IRSp53 plays a significant role in sensing and generating membrane curvature. IRSp53 anchors to lipid raft regions of the membrane via binding to the phospholipid PI(4,5)P2, thereby linking the plasma membrane to the actin cytoskeleton.

Full-length IRSp53 not only contributes to shaping membrane curvature but also influences actin polymerization through interactions with actin-regulatory proteins. Expression of IRSp53 has been observed in numerous mammalian tissues and cell types, particularly in neurons. Certain alleles of IRSp53 have been associated with neurological disorders, including alterations in dendritic spine density, attention deficits, and hyperactivity. Furthermore, IRSp53-knockout mice exhibit impairments in learning, memory, and synaptic plasticity.

1.4. Glycosylated and glycated proteins

Protein glycosylation and glycation are two distinct chemical processes that result in different biological effects.

Glycosylation is a post-translational modification and an enzymatic process during which sugar moieties, such as glucose or mannose, are specifically attached to a protein. Glycosylation influences protein-protein interactions and thereby affects the biological activity and physicochemical properties of proteins, including their solubility and stability. Two main types of glycosylation can be distinguished: O-linked and N-linked glycosylation.

In contrast, *glycation* is a non-enzymatic process in which reducing sugars react with free amino groups of proteins. This process is referred to as the Maillard reaction, and depending on the reactants and the reaction conditions, a wide variety of reactive compounds can be formed, resulting in considerable heterogeneity of glycation end products. Over time, the initial products undergo rearrangement and further transformation, leading to the formation of so-called AGEs (Advanced Glycation End-products). Reactive dicarbonyls and furan derivatives generated during the reaction are capable of irreversibly modifying proteins and, in some cases, forming

covalent cross-links between the side chains of different amino acids, thereby irreversibly altering protein structure.

1.5. sRAGE

The sensing of glycated proteins and other glycation end products is mediated by the receptor for advanced glycation end-products (RAGE). RAGE is a membrane protein that consists of an Ig-V (immunoglobulin variable-type) domain, two C-type immunoglobulin domains (C1 and C2), a hydrophobic transmembrane domain, and a cytoplasmic tail. Upon binding of AGEs, RAGE becomes activated, leading to increased production of cytokines, chemokines, and adhesion molecules, as well as enhanced oxidative stress. This process results in a positive feedback loop that increases RAGE expression and ultimately gives rise to a self-sustaining inflammatory response.

Soluble RAGE (sRAGE) is generated by alternative splicing or proteolysis, or by C-terminal cleavage, resulting in a form that lacks the transmembrane and cytosolic domains. sRAGE retains its affinity for glycation end products (AGEs) and therefore competitively inhibits the pro-inflammatory and pathological effects mediated by RAGE.

RAGE activation by AGEs is presumed to also influence the formation of NTs. In studies investigating inflammatory processes occurring during peritoneal dialysis, increased membrane nanotube formation was observed. During heat sterilization of glucose-containing dialysis solutions, AGEs are formed, which, through RAGE activation, lead to an increased number of NTs.

For studies involving sRAGE, a protein of sufficient purity was required. Glycosylation of RAGE is essential for its proper function. However, an appropriate glycosylation pattern can only be achieved through expression in mammalian cells. Mammalian cell-based expression systems are the most expensive; therefore, we chose to isolate the protein from native tissue. RAGE expression is restricted to specific tissues and organ systems, such as the kidney, the nervous system, and endocrine tissues, with particularly high expression observed in the lung. Due to the size of porcine and bovine lungs, multiple experiments can be performed using a single organ, representing a significant improvement compared to previous approaches relying on mouse lungs. Previous studies have demonstrated that RAGE is highly conserved among certain species, including humans, cattle, and pigs.

2. Objectives

During our investigations, the following objectives were defined:

1. To elucidate the role of the IRSp53 protein and its N-terminal I-BAR domain in the formation of NTs.
2. To examine whether the formation of filopodia and NTs occurs via identical mechanisms, and to determine how IRSp53 and its I-BAR domain influence these processes.
3. To perform a detailed microscopic analysis of the effects of the I-BAR domain and full-length IRSp53 protein on the morphology of NTs formed by different cell lines.
4. To study the growth of actin filaments in the presence of IRSp53 or the I-BAR domain using TIRFM and actin polymerization assays.
5. To develop a system that enables the regulation of membrane nanotube formation. For this purpose, NT formation was planned to be stimulated by glycated proteins, while inhibition of nanotube formation was intended to be achieved through the competitive effect of sRAGE.
6. To develop novel protocols that enable
   a. the isolation of sRAGE from porcine lung tissue with adequate yield and purity, and
   b. the efficient purification of ribose-glycated bovine serum albumin (RBSA).

3. Applied methods

3.1. Methods used for IRSp53 and I-BAR studies

3.1.1. Cell line maintenance and transfection

COS-7 kidney cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cultures were maintained at 37 °C in an incubator with 5% CO_2 under near-physiological conditions in Petri dishes equipped with specialized borosilicate glass windows. During experiments, a typical cell density of 25,000 cells/cm² was used.

A20 mature B lymphoma cells were cultured in an incubator at 37 °C in a 5% CO_2 atmosphere. The cells were maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, antibiotics, and 10% FBS. For subsequent experiments, a typical cell density of 3×10^5 cells/cm² was applied.

For live-cell experiments, cells were transfected with either mCherry-IRSp53 or mCherry-I-BAR plasmids. COS-7 cells were transfected using Lipofectamine 3000 reagent, while the A20 cell line was transfected by electroporation using an Amaxa Nucleofector IIb device, in both cases according to the manufacturers' instructions. In control experiments, a GFP-LifeAct plasmid encoding the LifeAct peptide fused to green fluorescent protein was used. In all experiments, 1 μg of plasmid DNA was used for IRSp53 and I-BAR, while 2 μg of plasmid was used for LifeAct. Samples were analyzed under live-cell conditions 16 hours after transfection.

3.1.2. Immunocytochemistry and cell staining

Cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, followed by permeabilization with 0.1% Triton X-100 and 5% bovine serum albumin (BSA) for 20 minutes at room temperature. Subsequently, cells were incubated with an anti-IRSp53 primary antibody (1:500) for 1 hour at room temperature, washed with 1× phosphate-buffered saline (PBS), and then incubated with a secondary antibody (1:1000; goat anti-rabbit Alexa Fluor 488). In certain cases, cells were additionally labeled with Alexa Fluor 488- or 561-phalloidin. Nuclei were stained using Hoechst dye (1:1000). Samples were mounted with VECTASHIELD antifade mounting medium and stored at 4 °C in the dark until microscopic analysis. The functionality of NTs was verified by Alexa Fluor 488-cholera toxin B labeling.

3.1.3. Laser Scanning Confocal Microscopy and Structured Illumination Microscopy (SIM)

The effects of the I-BAR domain and the IRSp53 protein on membrane nanotube formation were investigated using confocal and structured illumination microscopy (SIM). Confocal microscopy enables high-resolution imaging based on optical sectioning, allowing precise determination of the spatial localization of nanotubes. Super-resolution SIM imaging reveals additional morphological details and is therefore suitable for detailed analysis of protein localization and the structural characteristics of nanotubes.

The effects of transfection on cells and their protrusions were visualized using a Zeiss LSM 710 laser scanning confocal microscope with 63× magnification (oil immersion objective; N.A.: 1.4). Image acquisition and primary analysis were performed using Zen Black 2.1 SP3 and Zen Blue 2.3 software, followed by further image processing using ImageJ and, in certain cases, Imaris 8.2 software. The spatial relationship between IRSp53 and F-actin was examined by colocalization analysis within regions encompassing the entire length of the nanotubes. Protein colocalization along "stress fibers" was analyzed within elongated elliptical regions of

interest (ROIs) surrounding actin bundles. Colocalization coefficients M1 and M2 (Manders' coefficients) were calculated using Zen Black 2.1 SP3 software.

In the analysis of NTs and filopodia morphology, actin transfection was used as a control. Statistical analysis was performed in three independent experiments, with at least 800 cells visualized in each experiment.

3.1.4. I-BAR expression and purification

The I-BAR domain of IRSp53 was produced using a bacterial expression system, whereas full-length IRSp53 was obtained from MyBioSource. For protein expression, the cDNA encoding the IRSp53 I-BAR domain was cloned into the pGEX-4T2 vector (plasmid: glutathione S-transferase expression vector, 4T2) and expressed as a GST (glutathione S-transferase) fusion protein in *Escherichia coli* BL21 cells. The plasmid was kindly provided by Laura Machesky (University of Cambridge, Department of Biochemistry, Cambridge, UK). Bacteria were cultured in Luria Broth medium at 37 °C. Protein expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside and allowed to proceed overnight at 25 °C. Cells were then harvested by centrifugation and stored in 6 g aliquots at -80 °C until further use.

Purification of GST-tagged recombinant proteins was performed using glutathione affinity chromatography. Removal of the GST tag was achieved by on-column proteolytic cleavage with thrombin. The resulting tag-free target protein was subsequently purified from thrombin using a benzamidine affinity column. Typically, 3.5-4.0 mg/mL protein solution was obtained from 5-6 g of bacterial pellet.

3.1.5. Actin purification

Actin was isolated from rabbit back muscle and further purified by gel filtration using a Superdex 200 column. During the size exclusion chromatography (SEC) applied for purification (also referred to as gel filtration chromatography), proteins were separated based on their size and shape (Stokes radius). As is characteristic of SEC, proteins of larger size eluted earlier during the separation. This step enabled further purification of actin by removing smaller contaminants and aggregates.

Purified actin was stored in G-buffer (pH 7.8, 4 mM Tris-HCl, 0.1 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, and NaN_3 0.005% (w/v)). For TIRFM measurements, G-actin was labeled at Lys328 (lysine 328) with Alexa Fluor® 488 carboxylic acid succinimidyl ester (Alexa 488 NHS). To measure the kinetics of actin assembly, G-actin was labeled at Cys374 (cysteine 374) with N-(1-pyrenyl) iodoacetamide.

3.1.6. Total Internal Reflection Fluorescence Microscopy

Flow chambers were prepared using coverslips suitable for microscopy and incubated with 100 µL N-ethylmaleimide–treated myosin for 1 minute, followed by washing with 200 µL myosin buffer (pH 7.8, 0.1 mM $CaCl_2$, 1 mM dithiothreitol (DTT), 1,4-bis(sulfanyl)butane-2,3-diol, 0.2 mM ATP, 4 mM Tris-HCl, 500 mM KCl, 1 mM $MgCl_2$, and 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)) and subsequently with 200 µL 0.1% (w/v) BSA buffer (pH 7.8, 0.1 mM $CaCl_2$, 1 mM DTT, 0.2 mM ATP, 4 mM Tris-HCl, 100 mM KCl, 1 mM $MgCl_2$, 0.2 mM EGTA, and 0.1% (w/v) BSA). Immediately prior to measurement, 200 µL of TIRFM buffer was added to the protein solution. The TIRFM buffer consisted of F* buffer (pH 7.8), supplemented with 0.5% (w/v) methylcellulose, 0.5% (w/v) BSA, 50 mM 1,4-diazabicyclo[2.2.2]octane, and 100 mM DTT. The F* buffer (pH 7.8) contained 0.1 mM $CaCl_2$, 1 mM DTT, 0.2 mM ATP, 4 mM Tris-HCl, 50 mM KCl, 1 mM $MgCl_2$, and 0.2 mM EGTA. Free G-actin polymerization in the presence or absence of I-BAR/IRSp53 was investigated in flow chambers prepared as described above. I-BAR/IRSp53 and G-actin were mixed at various ratios in TIRFM buffer (0.5 µM actin, including 10% Alexa Fluor 488 NHS labeled G-actin). I-BAR was applied at 0.1×, 60×, and 120× molar ratios relative to actin, while IRSp53 was mixed with actin at 0.5× and 10× concentrations. Images were acquired every 10 seconds using a CCD camera with a laser-based (491 nm) TIRFM module mounted on an inverted microscope equipped with an apochromatic oil-immersion objective (APON TIRF 60×, N.A.: 1.45). TIRFM images were analyzed using Fiji software. The same region of interest (724 × 724 pixels) was selected in all images to eliminate intensity variations at the edges. After background subtraction (noise filtering radius of 50 pixels using the “sliding paraboloid” setting), a threshold of 1% was applied. To exclude residual background noise or irrelevant small objects, a minimum size of five pixels was defined during analysis. Processed images were compared with raw images, and manual corrections were applied when necessary to minimize discrepancies.

3.1.7. Actin polymerization test

During the polymerization assay, 2.5 µM G-actin labeled with 5% pyrene iodoacetamide was used and polymerized in the presence of different concentrations of I-BAR, or actin alone as a control, by the addition of 2 mM $MgCl_2$ and 100 mM KCl. Changes in pyrene fluorescence intensity were monitored using a fluorimeter (Safas Xenius FLX). A slit width of 3 nm was applied for excitation and 5 nm for emission. Excitation and emission wavelengths were set to λ_{ex} = 365 nm and λ_{em} = 404 nm, respectively, with a detector voltage of 720 V. Due to the low

concentration of the full-length recombinant IRSp53 protein, polymerization assays could not be performed with this protein.

Samples were mixed separately for each concentration immediately before measurement, polymerization was initiated in the cuvette, and fluorescence recording was started within a few seconds.

3.1.8. General experimental conditions

Confocal images were acquired from live cells under controlled environmental conditions (37 °C, 5% CO_2 atmosphere), and optical sectioning along the Z-axis was applied to clearly distinguish NTs.

All TIRFM and actin polymerization measurements were performed at 20 °C. In our experiments, Mg^{2+}-ATP–actin was used. For this purpose, Ca^{2+} bound to the actin monomer was exchanged for Mg^{2+} by the addition of 200 µM EGTA and 50 µM $MgCl_2$, with samples incubated for 5 minutes at room temperature. Data were obtained from at least three independent experiments.

3.1.9. Statistics

The relative frequencies of NTs and filopodia were calculated from at least three independent experiments, analyzing a minimum of approximately 800 cells per sample. Image analysis was performed using ImageJ/Fiji and Imaris 8.2 software, while statistical analyses, including hypothesis testing (Student's t-test or Mann-Whitney test), were conducted using Origin 2020 or IBM SPSS Statistics software. When tests of normality rejected a normal distribution, the Kruskal–Wallis test was applied (or, in the case of normal distribution, analysis of variance (ANOVA)) to compare individual treatments with the control. These latter two tests were complemented by post hoc analyses using Tukey and Bonferroni comparisons. The Mann-Whitney U test (or, in the case of normal distribution, Student's t-test) was applied to avoid type II errors and to confirm significance suggested by the Kruskal–Wallis analysis. The level of statistical significance was set at $p < 0.05$.

Regression analysis was performed to assess whether a linear relationship exists between the number of NTs and the number of cells present within the field of view. The strength of the fit was characterized by the correlation coefficient, while the coefficient of determination was used to express the proportion of variance in the dependent variable explained by the model.

3.2. Methods for sRAGE purification and analysis

3.2.1. Dissection of porcine lung

Cartilaginous parts were removed from porcine (*Sus scrofa*) lung tissue, and ten grams (wet weight) of soft tissue were homogenized in 200 mL of lysis buffer. The lysis solution was sonicated on ice, followed by centrifugation of the lysate at 30,000 rpm.

3.2.2. Selective precipitation of haemoglobin

Foaming and high viscosity of the lysate hindered chromatographic separation; therefore, selective precipitation was applied prior to purification to remove haemoglobin using a 0.5 M zinc sulphate solution. The resulting precipitate was removed by ultracentrifugation, after which the pH was adjusted to 9.0. As a result of the pH shift, excess zinc precipitated in the form of a zinc-Tris complex. This precipitate was removed by repeated ultracentrifugation. Subsequently, Mn^{2+} and Ca^{2+} ions were added to the solution at neutral pH (7.0).

3.2.3. Concanavalin A chromatography

Concanavalin A (Con A) affinity chromatography is an effective method for the purification of glycosylated proteins, including sRAGE, which contains two N-linked glycosylation sites. Concanavalin A is a lectin isolated from the seeds of *Canavalia ensiformis* that specifically binds carbohydrates and glycosylated proteins. Due to its metalloprotein nature, Con A adopts a closed conformation in the presence of Mn^{2+} and Ca^{2+} ions, which is essential for its carbohydrate-binding activity.

The immobilized Con A-containing stationary phase is capable of selectively binding glycosylated components of the lysate. After removal of non-binding proteins, the target protein is eluted competitively using methyl-α-D-mannopyranoside, which can be readily removed during subsequent purification steps.

3.2.4. Heparin chromatography

The protein solution obtained after Con A chromatography was loaded onto a 5 mL HiTrap heparin column which had been previously equilibrated with 20 mL of buffer A (pH 7.5, 50 mM NaCl, 20 mM Tris-HCl). For gradient elution, buffer B was used (pH 7.5, 1 M NaCl, 20 mM Tris-HCl).

All buffers were filtered through cellulose acetate membranes with a pore size of 0.22 µm. The sample was loaded onto the column using a peristaltic pump at a flow rate of 0.5 mL/min. Separation was performed by gradient elution, and fractions of 1.2 mL were collected. Samples

were taken at different points of the chromatogram, and the protein content of the fractions was analyzed by SDS–PAGE.

3.2.5. Hydrophobic Interaction Chromatography (HIC)

The sRAGE-containing fractions collected during heparin chromatography were pooled, and ammonium sulphate was added in small portions to reach a final concentration of 1 M. The Capto Butyl ImpRes column was washed with HIC-A buffer (pH 7.0, 1 M NH_4Cl, 20 mM Tris-HCl), after which the sample was injected onto the column. Elution was initiated with 100% HIC-A buffer, followed by application of a linear gradient using HIC-B buffer (pH 7.0, 5 mM $CaCl_2$, 20 mM Tris-HCl). During separation, 1 mL fractions were collected. Samples were taken at each step and analyzed by SDS–PAGE to assess protein composition.

Fractions deemed appropriate based on gel analysis were pooled and dialyzed overnight against 2 × 2 L phosphate-buffered saline (PBS; pH 7.2, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) using a cellulose membrane dialysis tubing. The dialyzed protein was concentrated using centrifugal concentrators (10 kDa molecular weight cutoff) at 3000 × g to a final volume of 500 µL. Samples were aliquoted into 50-100 µL portions, snap-frozen in liquid nitrogen, and stored at −80 °C until use.

3.2.6. Protein identification by Mass Spectrometry

Mass spectrometric analysis of our samples was performed at the Proteomics Laboratory of the HUN-REN SzBK (Hungarian Research Network, Szeged Biological Research Centre) and the HCEMM (Hungarian Centre of Excellence for Molecular Medicine). The purified protein was dialyzed against 2 × 2 L PBS to remove ammonium salts, followed by separation on 15% SDS–PAGE and visualization by Coomassie staining. Three 2 × 2 mm gel pieces were excised from different positions of the gel. The three gel slices were placed into microcentrifuge tubes and transported to Szeged while kept moist in purified water.

Samples were reduced with DTT, alkylated with iodoacetamide, and subsequently digested with trypsin (4 hours at 37 °C). After extraction, the digested proteins were dried and resuspended in 30 µL of 0.1% formic acid, from which 5 µL aliquots were subjected to tandem mass spectrometry (HPLC–MS/MS) analysis. Peptides were fragmented by collision-induced dissociation in a linear ion trap (itCID), and the accurate masses of the fragments were determined using the Orbitrap analyzer of a Waters nanoAcquity LTQ–Orbitrap Elite MS system.

Raw data were processed using Proteome Discoverer software, and database searches were performed using an in-house ProteinProspector database. The presence of contaminating proteins alongside the target protein was assessed using the SwissProt database (version 2019.06.12). However, due to the incompleteness of the porcine database, analysis was subsequently performed using the UniProt database, employing both the *Homo sapiens* and *Sus scrofa* datasets.

3.2.7. BSA glycation

A 10 mL glycation buffer solution was prepared (pH 7.4, 20 mM Tris-HCl, 10 mg/L BSA, and 1 M ribose) and sterile-filtered through a 0.22 μm syringe filter under laminar flow conditions. The solution was transferred into a sterile 15 mL Falcon tube, and the closed tube was hermetically sealed with parafilm to maintain sterility. The sample was then incubated at 37 °C for 7 days to allow the glycation process to proceed. As a result of glycation, a faintly brown-colored solution was obtained. The solution was subsequently concentrated to a final volume of 2.5 mL using a 30 kDa molecular weight cutoff centrifugal concentrator tube.

3.2.8. Glycated protein purification

Unreacted BSA was separated from its glycated form using an XK 16/20 column packed with a 20 mL Q Sepharose anion-exchange stationary phase. The starting buffer consisted of 20 mM Tris base (pH 9.0) and 5 mM NaCl, while gradient elution was performed using 20 mM Tris base (pH 9.0) containing 2 M NaCl. Separation was carried out using gradient elution.

During purification, 1.5 mL fractions were collected, and the glycated fractions were concentrated to a final volume of 2.5 mL using a 30 kDa molecular weight cutoff centrifugal concentrator. Buffer exchange to PBS was performed using a Sephadex G-25 stationary phase. Detection was carried out at 280 nm.

3.2.9. Native and SDS-gel electrophoresis

SDS-PAGE gels contained 50 μL of 20% (w/v) sodium dodecyl sulphate (SDS), while SDS was present in the running buffer at a concentration of 1 g/L. The composition of the separating gel was as follows: 0.4 M Tris-HCl, 6.8% / 0.2% (w/v) acrylamide/bis-acrylamide, 0.05% (w/v) ammonium persulphate, and 0.5% (v/v) N,N,N′,N′-tetramethyl ethylenediamine (TEMED). The stacking gel contained 0.1 M Tris-HCl, 4.0% / 0.1% (w/v) acrylamide/bis-acrylamide, and 0.07% (w/v) ammonium persulphate.

In our experiments, neither the gel nor the running buffer contained SDS during native gel electrophoresis. A tenfold concentrated running buffer stock was prepared containing 25 mM Tris-HCl and 200 mM glycine, which was diluted tenfold immediately before use.

During native gel electrophoresis, bromophenol blue dye was used to monitor sample migration. A threefold concentrated stock solution was prepared and added to the samples at a 1:3 ratio. The stock solution contained 240 mM Tris-HCl (pH 6.8), 30% glycerol, and 0.03% bromophenol blue. For preparation of the stock solution, a 0.4% (w/v) bromophenol blue solution made in high-purity deionized water was used.

Coomassie Blue staining was applied for protein visualization in both SDS-PAGE and native PAGE. Gels were first fixed in a solution containing 50% (v/v) methanol and 10% (v/v) acetic acid, followed by staining with 0.1% (w/v) Coomassie Blue R350 in 20% (v/v) methanol and 10% (v/v) acetic acid. Destaining was performed using a solution of 50% (v/v) methanol and 10% (v/v) acetic acid, and gels were subsequently stored in 5% (v/v) acetic acid solution.

3.2.10. Investigation of the effect of sRAGE on HEK-293 cells using confocal microscopy

The HEK-293 cell line was cultured in phenol red-free MEM Alpha (α-minimum essential medium) supplemented with 10% FBS at 37 °C in a 5% CO_2 atmosphere, while maintaining approximately 80% confluence. Microscopic imaging was performed using six-channel microscope slides. Cells were washed with PBS and detached from the culture vessel using 0.25% trypsin-EDTA, followed by centrifugation in PBS (1000 rpm) and resuspension in culture medium. From the resulting suspension, 54,000 cells were seeded into each channel of the slide.

After cell attachment (approximately 1 hour), the medium was replaced with either protein-containing medium used in the experiment or, in control conditions, the standard culture medium. To examine differences between glycated and non-glycated BSA, 5 µM BSA or 5 µM RBSA was added to the control medium. In sRAGE experiments, the control medium contained 5 µM sRAGE. Following overnight incubation, cells were labeled as described in Section 3.1.2 (Immunocytochemistry and Cell Staining), with actin stained using Alexa Fluor 561 phalloidin and nuclei stained with Hoechst dye. Confocal microscopy was performed using a Zeiss LSM 710 microscope equipped with a 20× objective (Plan-Apochromat, N.A.: 0.8, M27). The slide channels were visualized in tile scan mode, and when cells were distributed across multiple focal planes, Z-stack acquisition was applied to enable optical sectioning.

Cell counting was performed using Fiji ImageJ software. Images were contrast-enhanced using automatic thresholding based on cell density, applying the Brensen and Phansalkar methods, occasionally with a radius of 15 pixels and the Contrast option enabled. After conversion of images to binary format, gaps caused by missing pixels were filled using the “Fill Holes” function, and adjacent nuclei were separated using the watershed algorithm. The resulting images were analyzed using the Particle Analyzer function. Threshold values were varied between 50, 100, and 150 pixels. The lowest threshold was selected at which a maximum of 50 nuclei required manual correction due to background interference. Generated masks were manually inspected, and if more than 50 nuclei were miscounted, the analysis was repeated using a higher threshold. When fewer than 50 errors were present, results were corrected manually.

4. Results

4.1. Investigation of IRSp53 and I-BAR

4.1.1. Endogenous expression of IRSp53 and its presence in membrane nanotubes

Using confocal microscopy, endogenous expression of IRSp53 was detected in COS-7 and A20 cells, as well as in their membrane nanotubes. In A20 cells, IRSp53 exhibited a homogeneous intracellular distribution, with enrichment observed beneath the plasma membrane.

4.1.2. Colocalization of IRSp53 and actin

IRSp53 and filamentous actin colocalized beneath the plasma membrane, in the cytoplasm, and in NTs. The strength of colocalization was characterized using Manders’ coefficients. The degree of colocalization differed between the two cell types; however, in NTs, strong overlap was observed along the entire length of the tubes in both cell lines.

4.1.3. Effects of transfection with IRSp53 and the I-BAR domain on COS-7 cell morphology

The aim of these experiments was to elucidate how the IRSp53 protein and the I-BAR domain influence the morphology of filopodia and NTs in COS-7 cells. Overexpression of both I-BAR and IRSp53 affected the morphology of cellular protrusions. Compared to control cells, overexpression of either the I-BAR domain or full-length IRSp53 significantly increased the number and length of filopodia, with a more pronounced effect observed in the case of full-length IRSp53. In addition, both proteins increased the frequency of NTs and the number of their branches, while simultaneously reducing nanotube diameter. Overexpression of IRSp53 resulted not only in an increase in the length of nanotubes but also in the length of their

branches. Based on fluorescence intensity measurements, both I-BAR and IRSp53 showed local enrichment at specific sites within NTs. At these enrichment sites, nanotubes typically exhibited bending and subsequently continued in a different direction.

4.1.4. Effects of transfection with IRSp53 and the I-BAR domain on A20 B lymphoma cells

In A20 cells, transfection with IRSp53 and the I-BAR domain significantly increased the number and length of filopodia compared to control cells. Overexpression of I-BAR produced a more pronounced effect, similar to that observed in COS-7 cells.

In contrast, NT formation was not markedly affected by overexpression of either protein: neither the frequency nor the length of NTs changed significantly. Expression of the I-BAR domain led to the formation of thinner NTs, and although both proteins increased NT branching, the low incidence of these structures precluded statistically meaningful evaluation of branch length. Unlike in COS-7 cells, accumulation of the overexpressed proteins within NTs was only minimally observed in A20 cells.

4.1.5. Effects of IRSp53 and the I-BAR domain on actin polymerization

The effects of the IRSp53 protein and the I-BAR domain on actin polymerization were investigated using TIRFM and fluorescent actin polymerization assays under various sub- and super-stoichiometric concentrations. Under control conditions (0.5 μM actin), an average of 85 ± 55 actin filaments were formed. At a low I-BAR concentration relative to actin (actin:I-BAR = 10:1), the number of filaments increased slightly (100 ± 53), while their average length increased markedly (from 15.67 ± 4.27 μm to 19.08 ± 5.0 μm). At a higher, yet still sub-stoichiometric I-BAR concentration (actin:I-BAR = 5:1), the number of filaments decreased significantly (22 ± 14). In contrast, at high I-BAR concentration (60 μM I-BAR, actin:I-BAR = 1:120), the number of filaments increased dramatically (450 ± 150), while their length decreased substantially, with predominantly actin nuclei being observed.

Pyrene-labeled actin polymerization assays (2.5 μM actin, 5% pyrene) confirmed that at high I-BAR concentration (actin:I-BAR = 1:36), actin nucleation is accelerated, whereas at low concentration (actin:I-BAR = 1:10), polymerization kinetics do not differ from control conditions.

Analysis of the effects of IRSp53 revealed that statistical evaluation of TIRFM images showed that at sub-stoichiometric IRSp53 concentration (0.25 μM IRSp53, actin:IRSp53 = 2:1),

predominantly medium-length filaments were formed, whereas at high IRSp53 concentration (5 μM IRSp53, actin:IRSp53 = 1:10), the number of actin nuclei increased significantly.

Overall, our results demonstrate that both the I-BAR domain and the IRSp53 protein accelerate actin filament polymerization by promoting the formation of actin nucleation seeds.

4.2. Results related to sRAGE purification and BSA glycation

The mechanism of NT formation can be appropriately studied in a system in which nanotube formation can be experimentally regulated. One such approach is provided by activation of RAGE with glycation end products and its competitive inhibition by the sRAGE protein. Development of this experimental system requires the production of sRAGE and a glycated protein (RBSA in our case) with sufficient purity.

Based on phylogenetic analysis, RAGE proteins from pig (*Sus scrofa*) and cattle (*Bos taurus*) exhibit the highest similarity to human RAGE; therefore, the protein was isolated from porcine lung tissue.

4.2.1. Removal of haemoglobin contamination from porcine lung homogenates

sRAGE protein was isolated from porcine lung tissue; however, following homogenization, the resulting high-protein-content, red, and highly viscous solution did not lose its viscosity even after DNase treatment and, due to its instability, was unsuitable for chromatographic separation. Owing to the blood content of the tissue, the lysate obtained during homogenization contained substantial albumin and haemoglobin contamination, which led to pronounced foaming and promoted precipitation of the purified protein.

Therefore, haemoglobin was removed by zinc treatment: the addition of zinc ions to the solution induced denaturation of haemoglobin, which precipitated at pH values above 8.0. This effect was confirmed by SDS-PAGE and absorbance measurements. Based on control experiments performed with purified sRAGE, zinc treatment did not result in sRAGE loss and proved to be an effective method for selective removal of haemoglobin.

4.2.2. Concanavalin A chromatography

To isolate the biologically active, N-linked glycoform of sRAGE, Con A affinity chromatography was applied, as this glycoform is required for proper ligand-binding capacity of the protein. Concanavalin A specifically binds glycoproteins containing mannose and glucose side chains, thereby enabling selective separation of active sRAGE from inactive forms and other proteins.

Following zinc ion treatment, the stability and viscosity of the solution allowed direct injection of the sample onto the column without protein precipitation or an increase in backpressure. The presence of calcium and manganese ions required for Con A binding was ensured at pH 7.0, thereby preventing loss of affinity of the stationary phase.

During purification, non-glycoproteins were efficiently removed, resulting in a substantial increase in sample purity. The efficiency of separation and the purity of the collected fractions were confirmed by SDS-PAGE analysis.

4.2.3. Heparin chromatography

Heparin affinity chromatography was applied as an additional purification step, exploiting the specific binding of heparin to the extracellular domain of RAGE. The highly sulphated structure of heparin, composed of repeating disaccharide units, provides not only affinity-based binding but also weak ion-exchange properties, thereby enhancing separation efficiency.

During purification on the heparin column, several contaminating proteins were successfully removed, as confirmed by SDS-PAGE analysis. Removal of remaining impurities required further purification, which was achieved by HIC, taking advantage of the high salt concentration resulting from gradient elution.

4.2.4. Hydrophobic Interaction Chromatography (HIC)

The sRAGE protein does not contain the apolar transmembrane domain; however, it possesses hydrophobic surface regions. The principle of HIC method is based on the phenomenon that, in a mobile phase with high ionic strength, proteins orient their hydrophobic surfaces toward the apolar stationary phase and thereby bind to it. The sample eluted from the heparin column already exhibited a high salt concentration; therefore, binding to the butyl side chains of the HIC column could be achieved with the addition of only a minimal amount of ammonium sulphate. The interaction with the stationary phase is reversible, allowing proteins to be eluted by decreasing the salt concentration of the mobile phase. The efficiency of separation, however, is determined by the accessibility of hydrophobic regions on the protein surface. If these regions become buried within the protein interior due to conformational rearrangements, the efficiency of HIC separation may be significantly reduced.

SDS-PAGE analysis was complicated by differences in ionic strength among the collected fractions, which (together with the weaker SDS-binding properties of glycoproteins) resulted in an apparent shift in molecular weight. In the sample applied to the column containing 1 M ammonium sulphate, sRAGE appeared as a band of approximately 60 kDa on the gel.

During HIC, additional contaminating proteins were successfully removed: a ~50 kDa protein eluted at the beginning of the gradient, while a ~70 kDa component appeared after elution of the target protein at lower salt concentrations.

4.2.5. Identification by HPLC–MS/MS

The sample purified by HIC was analyzed by coupled HPLC–MS/MS following tryptic digestion. Based on the results, the purified protein did not contain significant contamination; only minimal keratin contamination was detected, along with low amounts of porcine chitinase-3-like protein 1 and prothrombin.

Isoforms of the target protein were not detected by this method; however, the porcine RAGE protein (UniProt accession: A0A4X1V556) was unambiguously identified. Exclusively peptides corresponding to the extracellular domain were detected, whereas peptides derived from the transmembrane region were absent, confirming that the sample contained the cleaved, soluble form of the protein, sRAGE. In addition, a RAGE variant exhibiting high sequence similarity (B9TSR6) was also suggested in the sample.

4.2.6. Ribose-mediated glycation of bovine serum albumin

Glycated proteins are typically prepared by incubation with glucose, and the process can be accelerated by increasing temperature. Ribose is significantly more reactive in glycation reactions than D-glucose, as it exists in solution at a much higher proportion in the open-chain (aldehyde-containing) form. Due to the instability of its five-membered ring, ribose more readily reverts to its reactive open-chain form, enabling faster and more extensive binding to proteins compared to the more stable ring structure of D-glucose. During glycation, the isoelectric point of the protein is altered; therefore, residual reactants and reactive dicarbonyl compounds were separated from the glycated protein by anion-exchange chromatography, followed by buffer exchange of the high-salt eluate to PBS using size-exclusion chromatography. During the 7-day glycation process, a characteristic brownish discoloration developed, which was not observed in the ribose-free control and intensified further during concentration. During separation on the anion-exchange column, unreacted ribose, reactive carbonyl compounds, and the pigments formed from them eluted in the early fractions, whereas glycated BSA eluted only at the end of the gradient as brown-colored fractions. The glycated fractions were pooled and concentrated to a final volume of 2.5 mL using a 30 kDa molecular weight cutoff centrifugal concentrator.

4.2.7. Analysis of sRAGE binding by Native PAGE

During native gel electrophoresis, mixtures of sRAGE, RBSA, and non-glycated BSA at different ratios were examined after overnight incubation at 4 °C. Under native PAGE conditions, proteins retain their native conformation, allowing assessment of the affinity between interacting proteins.

RBSA and sRAGE migrated as distinct bands with different mobilities when analyzed individually on native gels. This pattern was also observed for their 1:1 mixture; however, an additional, more slowly migrating band corresponding to a protein complex also appeared. Increasing the proportion of RBSA further enhanced the intensity of the complex band. Complex formation was also clearly detectable when sRAGE was present in excess.

In contrast, no complex formation was observed when sRAGE was incubated with non-glycated BSA, indicating that both sRAGE and the glycated protein were functionally intact and that a specific interaction occurs between them.

4.2.8. Effects of sRAGE and glycated BSA on NT formation in HEK-293 cells

Based on previous experiments demonstrating that activation of RAGE induces NT formation, we investigated whether RBSA is capable of eliciting this effect and whether nanotube formation can be inhibited by the application of sRAGE. NT formation was analyzed in HEK-293 cells by confocal microscopy under control conditions (medium alone) or in the presence of non-glycated BSA, RBSA, sRAGE and sRAGE+RBSA.

Due to the compact arrangement of the cells, membrane nanotubes could only be visualized in regions of low confluence. Cell numbers were determined by nuclear staining, while NTs were identified by fluorescent labeling of F-actin. Based on these data, relative nanotube numbers were calculated for hypothesis testing.

The relationship between cell number and NT number was examined by regression analysis, which revealed a strong positive correlation in control samples. NT numbers increased proportionally with cell number as long as cell density permitted nanotube visualization. In control samples, the high correlation coefficient ($r = 0.98$) indicated a strong linear relationship, while the R^2 value (96.03%) demonstrated that the linear model provided an excellent fit to the data.

Regression analysis revealed a similarly strong linear relationship between cell number and NT number in both BSA- and RBSA-treated samples. The model explained 85.36% of the variation

in NT number for BSA-treated samples and 81.15% for RBSA-treated samples, indicating good model fit and a strong positive correlation comparable to the control condition.

Across control, BSA-, and RBSA-treated samples, a strong linear relationship was consistently observed between cell number and NT number, indicating that NT numbers increased proportionally with increasing cell number. In the presence of RBSA, a higher regression coefficient indicated increased NT numbers at all examined cell densities, while the slope remained similar to that of the control. This suggests that RBSA enhances NT formation.

In the presence of sRAGE, the effectiveness of the regression model decreased markedly. Whereas strong positive correlations between cell number and NT number were observed in control, BSA-, and RBSA-treated samples, only a moderate correlation was detected in the presence of sRAGE ($r = 0.64$), with the model explaining only 40% of the variation in NT number. When RBSA and sRAGE were applied at a 1:1 ratio, the relationship further weakened, and the regression was no longer statistically significant, indicating that in the presence of sRAGE, the effect of cell number on NT formation is substantially reduced. Under combined RBSA and sRAGE treatment, cell number no longer influenced NT abundance.

Hypothesis testing confirmed that both RBSA and sRAGE significantly affect NT formation. As the data did not consistently follow a normal distribution, non-parametric statistical tests were applied, revealing significant differences in relative NT numbers between control, RBSA-treated and sRAGE-treated cells. Post hoc analyses showed that cells treated with sRAGE alone or with sRAGE + RBSA exhibited significantly lower relative NT numbers compared to controls, whereas RBSA treatment resulted in a significant increase in NT number. No significant difference in relative NT numbers was observed between control and BSA-treated samples.

No significant difference was detected between the effects of sRAGE alone and sRAGE + RBSA (1:1). These samples contained significantly fewer nanotubes than control and BSA-treated cells, while RBSA treatment significantly increased NT formation. Collectively, these results demonstrate that RBSA promotes, whereas sRAGE inhibits, NT formation.

5. Discussion

5.1. Conclusion of IRSp53 and I-BAR results

During our experiments, we established that endogenously expressed cytoplasmic IRSp53 protein is present in NTs formed by both COS-7 and A20 cells. Full-length IRSp53 increased both the number and length of NTs and filopodia in both cell lines (COS-7 and A20). Cell line–specific differences were also observed: in COS-7 cells, overexpression of IRSp53 and the I-BAR domain exerted a more pronounced effect on NT number compared to A20 cells, which is likely attributable to differences in NT formation mechanisms between the two cell types.

Transfection of COS-7 and A20 cells with either IRSp53 or its I-BAR domain resulted in increased filopodia number and length in both cell lines. However, only full-length IRSp53 could induce comparable changes in both NT and filopodia numbers across the two cell lines. These findings indicate that both IRSp53 and its I-BAR domain play important roles in regulating NT dynamics. While the I-BAR domain is likely to primarily contribute to NT formation through its membrane-curvature–inducing properties, full-length IRSp53 appears to play a more decisive role in NT elongation.

We also observed thinning of NTs, which is presumably attributable to their increased growth dynamics. In addition, elevated concentrations of IRSp53 or the I-BAR domain led to enhanced NT branching, consistent with the membrane-curvature–inducing properties of the I-BAR domain.

Furthermore, our TIRFM-based analyses and actin polymerization assays demonstrated that both IRSp53 and its I-BAR domain possess actin-nucleating activity. According to our model, local accumulation of I-BAR and IRSp53 forms focal points that promote the growth of membrane protrusions and NTs, while IRSp53 ensures uniform distribution of actin filaments along the entire length of NTs. Actin nuclei formed as a result of the nucleating activity of IRSp53 provide new binding sites for actin filament elongation. Newly formed actin filaments reinforce the growing actin bundles. These molecular anchoring points establish the mechanisms that allow the number of actin filaments to remain approximately constant along the full length of NTs. Because IRSp53 dimers are anchored to the membrane, they also function as mechanical support points for actin filament bundle growth and for force generation along NTs.

5.2. Conclusion of the results related to sRAGE purification

We chose to isolate sRAGE from porcine lung tissue because the protein exhibits a highly conserved sequence, an appropriate molecular size, and a mammalian-type N-linked glycosylation pattern. Due to the high blood content of lung tissue, significant haemoglobin contamination was encountered during protein extraction, which was selectively precipitated using a zinc-containing buffer. As a result, a clarified solution was obtained that proved suitable for subsequent chromatographic purification steps.

Using a Concanavalin A column, glycosylated proteins were successfully separated from other proteins lacking carbohydrate side chains. sRAGE was then further efficiently purified by exploiting its affinity for heparin using heparin affinity chromatography, a process enhanced by the weak ion-exchange properties of the column. To remove remaining impurities, hydrophobic interaction chromatography was also applied. By employing a more complex purification strategy than those previously described in the literature, we were able to obtain 0.77–1.5 mg of highly purified sRAGE protein from 10 g of porcine lung tissue, which was subsequently identified by HPLC–MS/MS analysis.

As a ligand for sRAGE, RBSA was used and purified by ion-exchange chromatography following glycation to ensure appropriate purity. Binding of RBSA to sRAGE was confirmed by native PAGE. Based on these results, the obtained proteins proved suitable for investigating the regulation of NT formation in cell culture. Therefore, the effects of sRAGE and RBSA were examined in the HEK-293 cell line, which endogenously expresses the RAGE protein.

Compared to control conditions, treatment with non-glycated BSA did not result in a significant change in NT number. In contrast, the addition of RBSA to the culture significantly increased NT numbers relative to both the control and non-glycated BSA treatments. Treatment with sRAGE, however, led to a significant reduction in NT number, although complete inhibition was not observed. This finding is consistent with previous RAGE knockout studies, in which absence of the receptor resulted in reduced but not completely abolished nanotube formation.

Competitive inhibition of the RAGE receptor was investigated by combined application of sRAGE and RBSA. The inhibitory effect of sRAGE on NT number remained detectable even in the presence of equimolar RBSA.

Based on these results, we conclude that the glycated protein RBSA significantly enhances NT formation, whereas sRAGE exerts an inhibitory effect on nanotube formation. Thus, our

original objective was achieved, as we established an experimental system that enables controlled regulation of NT formation.

6. Presentation of new results:

6.1. Effects of IRSp53 and the I-BAR domain on NT formation

6.1.1. We demonstrated that the IRSp53 protein is endogenously expressed in COS-7 monkey kidney cells and A20 mouse B lymphoma cells, and that it is present in the membrane nanotubes formed by these cells.

6.1.2. We showed that both the IRSp53 protein and its I-BAR domain exert a significant effect on the morphology of the investigated cells.

6.1.3. We demonstrated that transfection with plasmids encoding IRSp53 or the I-BAR domain resulted in an increased number and length of filopodia in both COS-7 and A20 cell lines.

6.1.4. We observed a reduction in the diameter of the formed nanotubes in response to protein expression, which we attribute to their increased growth dynamics.

6.1.5. We found that overexpression of these proteins led to a significant increase in NT branching, particularly in the case of the I-BAR domain, which is known to possess strong membrane curvature–inducing properties.

6.1.6. Using TIRFM analyses and actin polymerization assays, we demonstrated that both the full-length IRSp53 protein and its N-terminal I-BAR domain exhibit actin-nucleating activity. Membrane-associated I-BAR and IRSp53 promote the formation of new actin filaments, thereby stimulating actin assembly and supporting not only the formation but also the elongation of cellular protrusions.

6.1.7. We demonstrated that IRSp53 colocalizes with actin filaments independently of the examined cell type and, through its membrane-binding capacity, may facilitate the structural organization of membrane nanotubes.

6.1.8. We proved that IRSp53 predominantly promotes the formation of NTs originating from actin filament-driven, filopodia-like protrusions. This mechanism is characteristic of COS-7 cells. This effect was not observed in A20 cells, which form their NTs through an alternative mechanism involving opposing directional movement of interacting cells.

6.1.9. We proposed a model according to which IRSp53 stabilizes the structure of forming membrane nanotubes not only through its membrane curvature-inducing

properties but also via its actin-organizing functions, thereby providing structural support for the growth of new actin filaments. By clustering into focal sites, IRSp53 stimulates the initial phase of actin polymerization (nucleation), promotes the formation of new actin filaments, and provides a physical link between the membrane and F-actin.

6.2. Production of sRAGE and its binding to glycated proteins

6.2.1. We developed a purification protocol that enables the isolation of high-purity sRAGE protein from porcine lung tissue. This approach significantly reduced the number of animals required compared to previously published protocols. Due to its size, a single porcine lung provided sufficient protein for multiple experiments. As part of this protocol:

6.2.1.1. We established an efficient method for removing haemoglobin, which otherwise prevents chromatographic separation, by selective precipitation using a zinc-containing buffer, without causing loss of sRAGE during the treatment.

6.2.1.2. We successfully applied hydrophobic interaction chromatography (HIC) for the purification of sRAGE for the first time, enabling the removal of contaminants present in the sample after tissue homogenization.

6.2.2. We developed a purification strategy distinct from previously published protocols, consisting of multiple chromatographic steps (affinity, ion-exchange, and hydrophobic interaction chromatography), which enabled the isolation of sufficient amounts of highly purified sRAGE protein from porcine lung tissue.

6.2.3. Using HPLC–MS/MS analysis, we demonstrated that the purified protein was identical to sRAGE.

6.2.4. We produced glycated BSA of sufficient purity to demonstrate the functional activity of sRAGE and to investigate its effects on the HEK-293 cell line.

6.2.5. We developed a protocol for the preparation of glycated BSA using D-ribose as the reducing sugar, which, due to its structural properties, allowed acceleration of the glycation process. Our measurements indicated that an adequate degree of glycation was achieved after only one week of incubation at 37 °C. We also established a protocol to separate residual reactants, by-products, and the glycated protein formed during BSA glycation. The uniqueness of this method lies in the fact that the final product was a glycated protein dissolved in a native buffer free

of reactive by-products, making it suitable for live-cell experiments. The speed of the technique is comparable to the fastest glycation protocols currently available.

6.2.6. Using native PAGE, we confirmed that sRAGE binds specifically and with appropriate affinity to glycated BSA, while no binding was observed to non-glycated BSA used as a control.

6.2.7. We investigated the effect of glycated BSA (RBSA) on the HEK-293 cell line. The protein proved to be functionally active, as it significantly increased the number of membrane nanotubes formed between cells, whereas non-glycated BSA did not induce any change compared to untreated control cells.

6.2.8. We demonstrated the functional activity of sRAGE in live-cell experiments, as its presence in HEK-293 cultures significantly reduced the number of membrane nanotubes. Although complete inhibition was not observed, the inhibitory effect of sRAGE was also evident in the presence of equimolar amounts of RBSA.

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