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Journal of Immunological Methods 260 (2002) 263–271

JIM
Journal of
Immunological Methods

www.elsevier.com/locate/jim

Immunopurification of Golgi vesicles by magnetic sorting

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Received 15 June 2001; accepted 1 November 2001

Abstract

We have designed a method that permits to isolate highly purified Golgi vesicles deprived of endoplasmic reticulum (ER), main contaminant of Golgi fractions. To this end, we prepared a rabbit polyclonal antibody against the cytosolic N-terminal oligopeptide of the enzyme heparan glucosaminyl *N*-deacetylase/*N*-sulphotransferase (HSST), a specific marker for Golgi apparatus. The Golgi localization of HSST was confirmed by indirect immunofluorescence microscopy. The antibody binding to Golgi vesicles was demonstrated by immunoelectronmicroscopy and allowed the immunopurification by magnetic sorting. Golgi vesicles subjected to purification by magnetic sorting showed the presence of HSST and p28, which is an integral membrane protein on the *cis*-Golgi also used as a specific Golgi marker. The purified material was devoid of calreticulin, a specific ER marker. This purification method will allow to improve studies requiring highly purified Golgi membranes such as identification of specific receptors and the electrophysiological characterization of Golgi membrane ion channels, which have been jeopardized up to now by ER membrane contamination.

Keywords: Golgi vesicles; Heparan glucosaminyl *N*-deacetylase/*N*-sulphotransferase; Immunopurification; Magnetic sorting

1. Introduction

The Golgi complex, an organelle present in all eucaryotic cells, plays a crucial role in the posttranslational modification of proteins, synthesis of proteoglicans, glycolipids and polysaccharide in plant cells. These biochemical processes are catalyzed by specific enzymes, which for optimal function require particular luminal ionic conditions such as low pH (Glickman et al., 1983; Berger and Roth, 1997) and high calcium concentration (Chanatt and Hutter, 1991; Carnell and Moore, 1994; Dürr et al., 1998). Little is known about the ion transport mechanisms present in the Golgi membrane. Studies using enriched fractions of the organelle have shown the presence in the organelle of an electrogenic H^+ active transport, a passive Cl^- transport (Glickman et al., 1983), an active calcium uptake (Taylor et al., 1997; Pinton et al., 1998; Rojas et al., 2000) and an IP_3-sensitive Ca^{2+} release (Surroca and Wolff, 2000).

The electrophysiological study of ionic channels present in the Golgi membrane is an utmost necessity for understanding the role that these ion transport proteins play in the maintenance of the luminal ionic

Abbreviations: BSA, bovine serum albumin; DABCO, diazabicyclo[2,2,2]octane; ELISA, enzyme-linked immunoassay; ER, endoplasmic reticulum; FCS, fetal calf serum; HSST, heparan glucosaminyl *N*-deacetylase/*N*-sulphotransferase; MACS, anti-rabbit IgG Microbeads; PAGE-SDS, polyacrylamide gel electrophoresis with sodium dodecylsulphate; PBS, phosphate-buffered saline; PFA, paraformaldehide; PMSF, phenylmethylsulfonyl fluoride.

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0022-1759/02/$ - see front matter
PII: S0022-1759(01)00546-4

environment. As for the other organelles, the electrophysiological characterization of Golgi channels by reconstitution in phospholipid bilayers requires the use of highly purified membrane preparations with minimal contamination by other organelle membranes. Different procedures for the isolation of Golgi complex fractions have been described mainly based on ultracentrifugation of microsomal fractions on continuous sucrose gradients (Leelavathi et al., 1970; Fleisher, 1983; Wibo et al., 1981). The main disadvantage of these methods is that the obtained Golgi fractions present some degree of contamination by endoplasmic reticulum (ER) membranes (4–8%). Using a modification of the method described by Leelavathi, which allows a further enrichment of the Golgi markers, Nordeen et al. (2000) identified an anion channel with a maximum conductance of 130 pS and six conductances that they attributed to the Golgi.

Due to their exquisite specificity and reliability, antibodies have been used as specific tools for the identification and localization of proteins in numerous techniques (Paul, 1999). In the present study, we have designed a method to specifically isolate Golgi vesicles (cytosolic side out) devoid of ER membranes by immunopurification. We selected the heparan glucosaminyl *N*-deacetylase/*N*-sulphotransferase (HSST) as a specific marker for Golgi, a 110-kDa glycoprotein type II membrane protein, which has been shown to be located to the *trans*-Golgi network (Orellana et al., 1994; Humphries et al., 1997). We prepared a polyclonal antibody against the HSST cytosolic N-terminal oligopeptide sequence: NH_2-Met-Pro-Ala-Leu-Ala-Cys-Leu-Cys-Arg-His-Leu-Ser-Pro-Gln-Ala-Lys-COOH, named *N*-HSST peptide. The HSST Golgi localization was confirmed by indirect immunofluorescence microscopy. Immunogold labeling showed the binding of this antibody to Golgi vesicles. Immunopurification by magnetic sorting showed enrichment of vesicles containing HSST detected by immunoblotting of the purified material. The purified Golgi fraction showed also the presence of p28, an integral membrane protein of the *cis*-Golgi (Subramamiam et al., 1996) and it was devoid of calreticulin, a specific ER marker (Michalak et al., 1999). This anti-*N*-HSST antibody will be a useful tool for further studies on membrane transport and the search for specific receptors present only in Golgi.

2. Materials and methods

2.1. Reagents and antibodies

PMSF, pepstatin A, aprotinin and leupeptin were obtained from Sigma (St. Louis, MO, USA). The anti-p28 monoclonal antibody was kindly provided by Dr. V.N. Subrimamiam, National University of Singapore. The goat anti-rabbit Polygold 15 nm was purchased from Polysciences (Warrington, PA, USA). The goat anti-rabbit IgG Microbeads and the super paramagnetic MACS Microbeads, 50 nm, were obtained from Miltenyi (Germany). The rabbit anti-calreticulin was from ABR Inc. NJ, USA. The goat anti-rabbit IgG-alkaline phosphatase, the FITC-conjugated anti-rabbit IgG, the complete and the incomplete Freund's adjuvant and Tween 20 were obtained from Pierce Chemical (Rockford, IL, USA). The *N*-HSST peptide was synthesized in BioSynthesis (Lewisville, Dallas/Fort, TX, USA). Glutaraldehyde was from Polysciences. The enhanced chemoluminescence Western blotting detection kit and the IL-hyperfilm autoradiographic film were from Amersham (Arlington Heights, IL, USA). HEP-2 cells were from Kallestad (USA). Blue Carrier, hemocyanin from *Concholepas concholepas* was provided by Biosonda (Santiago, Chile).

2.2. Isolation of Golgi vesicles

Rat liver Golgi membrane fractions were obtained with a modification of the protocol originally described by Leelavathi et al. (1970). Briefly, livers were minced and suspended in 80–120 ml of cold buffer containing 0.5 M sucrose, 100 mM KH_2PO_4, 5 mM $MgCl_2$, pH 6.7 and a mixture of protease inhibitors (PMSF, pepstatin A, aprotinin, and leupeptin). Homogenization was carried out by using a Tekmar Tissumizer at low speed for 45 s. The homogenate was then centrifuged at $600 \times g$ for 5 min at 4 °C. The supernatant (post-nuclear fraction, PNF) was collected, and loaded on top of a 1.3 M sucrose layer in a polyallomer tube. The gradient was centrifuged at $100{,}000 \times g$ for 60 min at 4 °C. This resulted in the formation of a supernatant and a membrane layer above the 1.3 M interphase. The supernatant was removed and two sucrose layers of 1.1 and 0.25 M were overlaid on the membrane layer. The gradient was centrifuged at $100{,}000 \times g$ for 60 min. Two main

membrane fractions were obtained: one at the 0.25–1.1 M sucrose interface and the other at the 1.1–1.3 M sucrose interface. These fractions, named Golgi- and ER-enriched fractions, respectively, were collected and diluted with cold distilled water to twice the volume. Both suspensions were then spun down at 100,000 × *g* for 60 min at 4 °C. The sediments were gently resuspended in 2 ml of buffer (0.25 M sucrose, 10 mM Tris–Cl, 1 mM $MgCl_2$, pH 7.5) with a glass-teflon homogenizer. Membrane vesicles were aliquoted and stored at − 70 °C.

2.3. Preparation and characterization of the anti-N-HSST rabbit antibody

2.3.1. N-HSST peptide coupling to carrier protein

The polyclonal antibody was prepared against the HSST cytosolic N-terminal sequence NH_2-Met-Pro-Ala-Leu-Ala-Cys-Leu-Cys-Arg-His-Leu-Ser-Pro-Gln-Ala-Lys-COOH, named *N*-HSST peptide. Lysine was added at the carboxy-terminal Ala to allow the binding to the carrier throughout both ends. The peptide *N*-HSST was coupled to Blue Carrier using the protocol described by Coligan et al. (1991) with minor modifications by Becker et al. (1998). Briefly, 5 mg of the carrier protein was dissolved in 2 ml of 0.1 M borate buffer pH 10 and 5 mg of *N*-HSST peptide in bidistilled water was slowly added followed by glutaraldehyde to a final concentration of 0.3%. The mixture was incubated for 2 h in darkness at 25 °C, then dialyzed against phosphate-buffered saline (PBS; 0.1 M NaCl, 2.5 mM KCl, 1.5 mM KH_2PO_4, 7 mM Na_2HPO_4, pH 7.2) at 4 °C.

2.3.2. Rabbit immunization

Two New Zealand female rabbits were immunized with *N*-HSST coupled to Blue Carrier. On day 1, they received a subcutaneous and intradermal injection of 150 μg of antigen in complete Freund's adjuvant. The same immunization was repeated but with incomplete Freund's adjuvant. Prior to the immunizations, rabbits were bled to obtain pre-immune control sera. After each injection, serum was collected to determine the humoral immune response to the antigen.

2.4. Enzyme-linked immunoassay

To determine the presence of specific antibodies against *N*-HSST peptide, a direct ELISA assay was performed using the peptide without the carrier protein, Golgi- or ER-enriched membrane fractions as antigen solutions (Crowther and Abu-Elzein, 1980). Five hundred nanograms of the peptide or 1250 ng of the crude Golgi or ER vesicles in PBS was bound to the wells of polystyrene plates and incubated overnight at 4 °C. After the antigen incubation, the plates were washed three times with a solution containing 0.02% Tween 20 in PBS and blocked with 1% bovine serum albumin (BSA) in PBS (BSA–PBS) for 1 h at room temperature in a humid atmosphere. Fifty microliters of twofold antibody dilutions in 1% BSA–PBS was added to each well, similar volumes of parallel dilutions of non-immune sera were added in a different row. After overnight incubation at 4 °C, the wells were washed as above. Fifty microliters of alkaline phosphatase anti-rabbit IgG 1:1000 in BSA–PBS was added and incubated during 30 min at 37 °C. The plates were then washed as above and developed during 30 min at 37 °C with 50 μl/well of pNPP 1 mg/ml in a buffer solution containing 0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5. The reaction was stopped with 50 μl of 3 N NaOH and the absorbance was read at 405 nm. Pre-immune serum from the experimental animals was used as control.

2.5. Immunocytochemistry

The indirect immunofluorescence procedure on commercial 12 well/slides ready to use slides containing HEP-2 cells was the following: cells were incubated overnight at 4 °C with 15 μl of different dilutions in 1% BSA in PBS of the anti-*N*-HSST serum, washed with 0.02% PBS–Tween and incubated during 30 min at 37 °C with FITC anti-rabbit IgG diluted 1:100 in BSA–PBS. After washing as above, the cells were mounted in 10% DABCO medium (45% glycerol, 5% PBS and 0.04% sodium azide). The preparations were observed in a Zeiss Inverted microscope equipped with epifluorescence or in a Zeiss Confocal microscope.

2.6. Immunoelectronmicroscopy

For immunogold labelling, vesicles of Golgi-enriched fractions at a protein concentration of 10 mg/ml were centrifuged to obtain a pellet, which was fixed overnight at 4 °C in 4% PFA 0.1% glutaraldehyde in

PBS pH 7.5. Samples were extensively washed in PBS, cryoprotected and frozen in liquid Nitrogen. Ultrathin cryosections were prepared and incubated with anti-HSS in 2% FCS/PBS for 2 h at room temperature followed by 1-h incubation in goat anti-rabbit Polygold. Sections were washed with 2% FCS/PBS, post-fixed (10 min) with 2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 and stained for 10 min with 0.2% uranyl acetate, 0.2% methyl-cellulose, and 3.2% polyvinyl alcohol and then examined in a Phillips CM10 electron microscope.

2.7. Immunopurification by magnetic beads

Enriched Golgi vesicles with a protein concentration of 10 mg/ml were used for immunopurification. One hundred micrograms of vesicles was used for 1 ml of final incubation volume. Several dilutions of the anti-HSS antibody were allowed to react with 100 μg of Golgi vesicles to select the best antibody dilution for purification. The Golgi vesicles were mixed with the appropriate dilution of the anti-HSS antibody in 2% FCS in PBS or the pre-immune serum as control. Fifty microliters of goat anti-rabbit IgG Microbeads (super paramagnetic MACS MicroBeads, 50 nm) was added as secondary antibody. The final mix was incubated for 2 h at 4 °C with smooth rocking. After the incubation, the whole mix was subjected to a magnetic field for 5 min. The supernatant was recovered, the bound vesicles were washed several times and finally released from the magnetic field and resuspended in 2% FCS in PBS. The washed beads containing the Golgi vesicles–anti-*N*-HSST complex were resuspended in 50 μl PBS and the protein concentration of unbound and bound fractions determined.

2.8. SDS-PAGE and immunoblot analysis

Samples were electrophoresed by using SDS-PAGE either 10% or 12% according to the Laemmli method (Laemmli, 1970). Gel loading was standardized to equal amount of protein as determined by the Lowry assay. Proteins were electrotransferred at 100 V for 1 h or 40 V overnight to nitrocellulose membranes and treated with rabbit anti-*N*-HSST, anti-calreticulin, or anti-p28 at the appropriate dilutions. The secondary antibody was either peroxidase-labeled anti-rabbit or peroxidase-labeled anti-mouse immunoglobulins. The membranes were developed using the enhanced chemiluminescence Western blotting detection kit (ECL) and exposed to Hyperfilm autoradiographic film.

3. Results

3.1. Antibody characterization

After the third immunization, a titer of about 1:5000 against *N*-HSST peptide in solid phase inmunoassay (ELISA) was obtained. For immuno-histochemistry, a working dilution of 1:1000 was used since the pre-immune serum showed low reactivity to the peptide at that dilution (Fig. 1A). To investigate

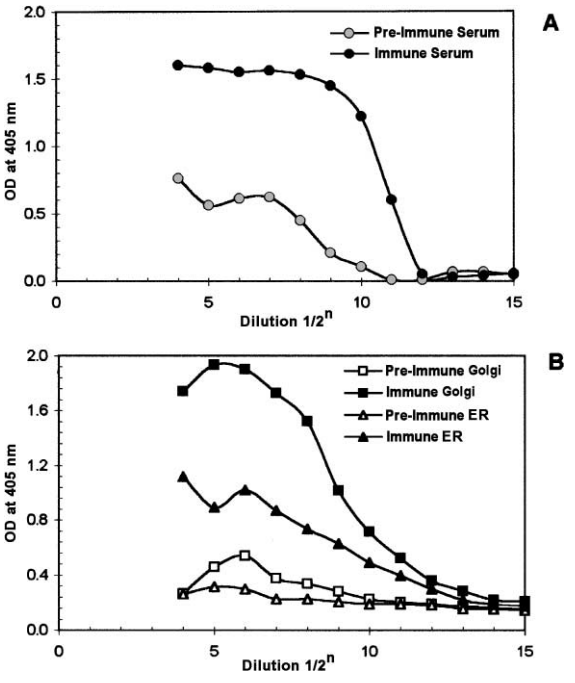

Fig. 1. Specificity of the humoral immune response of rabbit against *N*-HHST peptide. (A) Titration of serum from a rabbit immunized with *N*-HSST peptide, using a direct ELISA. The plates were activated with the peptide, and the binding of the specific antibodies to the antigen was determined using an anti-rabbit IgG labeled with alkaline phosphatase. The plate was revealed with pNPP solution as described in Materials and methods. Pre-immune serum was used as control. (B) Titration of anti-*N*-HSST peptide serum by a direct ELISA using Golgi and ER vesicles bound to the solid phase. Pre-immune serum was used as control.

the subcellular specificity of the anti-*N*-HSST serum, ELISA plates were coated with the enriched Golgi or ER vesicle fractions; the immune sera exhibited a strong reaction to Golgi and a weak reaction to ER (Fig. 1B). The above results indicated that the peptide design and the immunization protocol produced a high titer and specific sera.

3.2. Immunofluorescence

The immunolocalization of HSST done on HEP-2 cells by indirect immunofluorescence microscopy showed that this protein is confined to the perinuclear structures typical of Golgi (Fig. 2A). Similar immunofluorescence pattern was observed in Cos-7 and

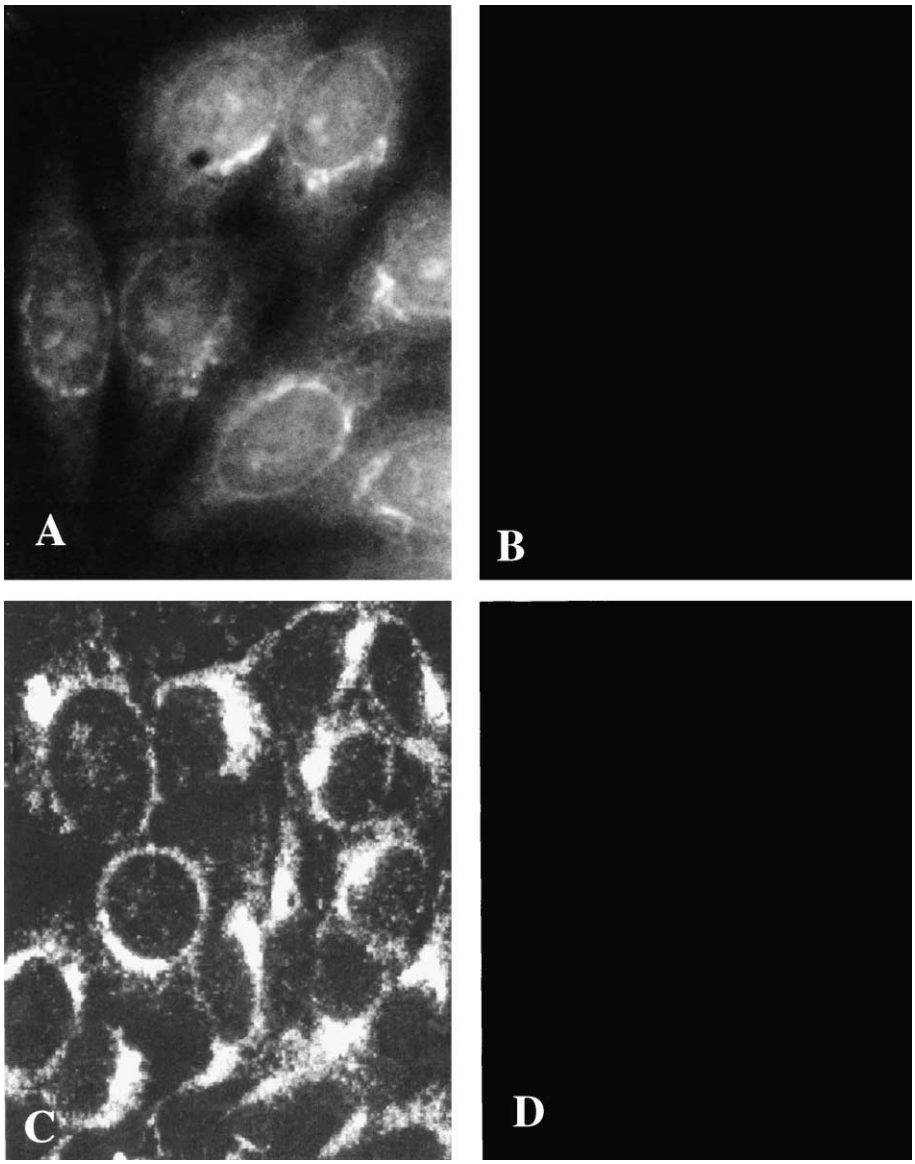

Fig. 2. Immunolocalization of the heparan sulphate transferase in HEP-2 cells. Cells were fixed and stained with anti-*N*-HSST (A) or anti-p28 antibody (C). Both antibodies gave similar patterns of staining reticulated membranes within the perinuclear Golgi complex. The pre-immune serum did not show any labeling (B, D).

MDCK cells (not shown). The Golgi localization was confirmed by comparison with a Golgi marker, the 28-kDA protein, p28, this monoclonal antibody was kindly provided by Dr. V.N. Subrimamiam (Fig. 2C). In each case, controls for background labeling with the secondary antibody gave negligible labeling, Fig. 2B and D.

3.3. Immunoelectron microscopy

To show that the antibody recognized the Golgi vesicles, they were treated with the anti-*N*-HSST antibody followed by the anti-rabbit Polygold for electronmicroscopy. Golgi vesicles treated with the anti-HSST antibody showed gold particles bound to membrane vesicles or included in clear or electron-dense vesicles (Fig. 3B, C, D) in contrast with the control samples, in which few gold particles are seen randomly distributed (Fig. 3A).

3.4. Immunopurification by magnetic beads and characterization of isolated material

Golgi vesicles treated with the anti-HSST followed by the goat anti-rabbit IgG Microbeads (MACS) subjected to a magnetic field retained a subpopulation of vesicles. This enriched fraction contained a protein recognized by the anti-HSST antibody by immunoblotting (Fig. 4, lane 6). This protein was not detected in untreated Golgi vesicles (lane 1, 100 μg; lane 2, 50 μg; lane 3, 25 μg), in the Golgi vesicles treated with the pre-immune serum (lane 5), neither in Golgi

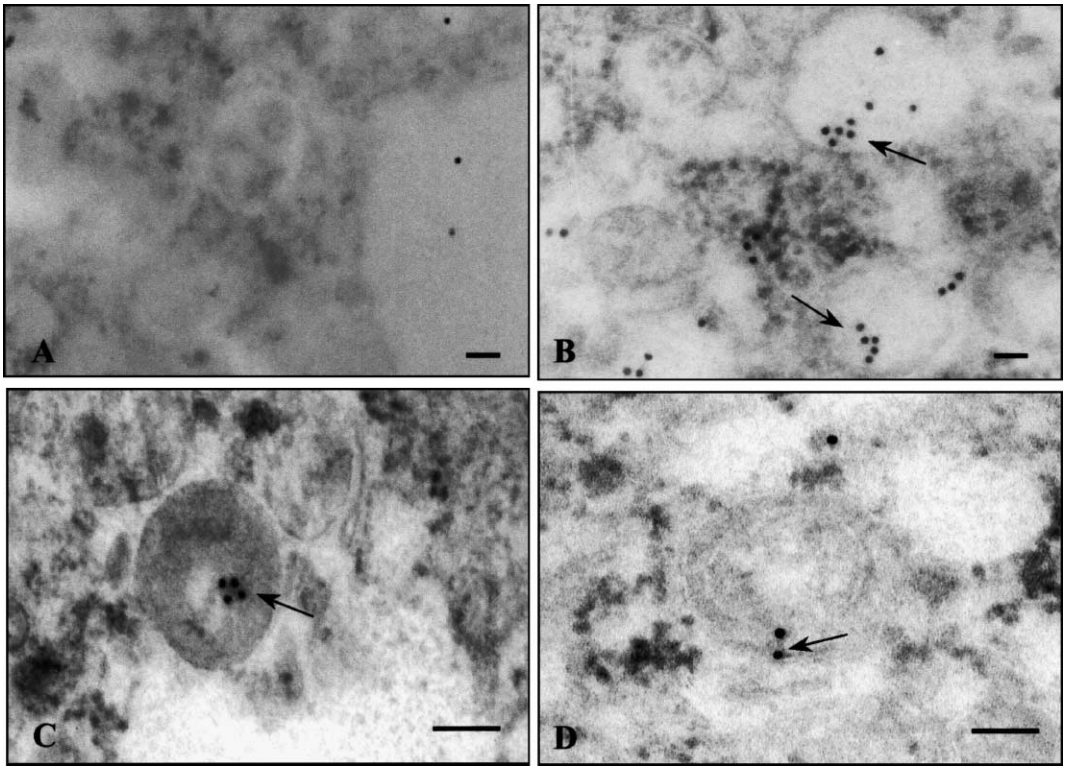

Fig. 3. Immunoelectron microscopy of Golgi vesicles. Control preparation incubated with the secondary antibody adsorbed on gold particles. Few particles are seen without specific localization (A). In contrast, Golgi vesicles incubated with the anti-HSST antiserum and gold particles coated with the anti-rabbit IgG showed that the gold particles were concentrated within vesicles (B and C) or bound to Golgi membrane (D). Bar represents 100 nm.

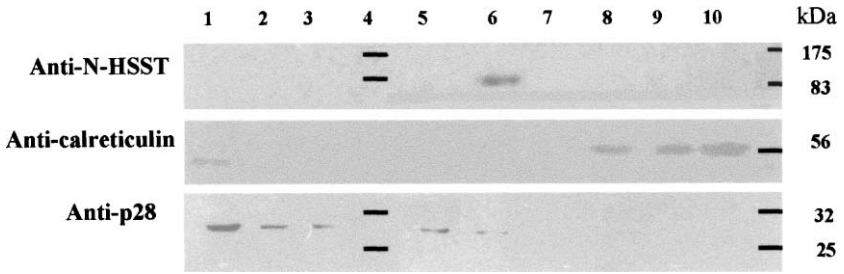

Fig. 4. Immunoblot of purified Golgi vesicle proteins separated by SDS-PAGE. Samples of each fraction were solubilized in SDS-PAGE sample buffer before running on a 10% polyacrylamide gel and transferred to nitrocellulose membrane and probed with anti-*N*-HSST, anti-calreticulin or anti-p28. Lanes 1, 2 and 3 were loaded with 100, 50 and 25 μg of untreated Golgi vesicles, lane 4 with molecular weight marker. Lane 5 shows Golgi vesicles treated with pre-immune serum and MACS separated by a magnetic field. Lane 6 shows Golgi vesicles treated with an anti-HSST antibody and MACS separated by a magnetic field. Lane 7 corresponds to the Golgi vesicles treated with an anti-HSST antibody and MACS, not retained by the magnetic field. Lanes 8, 9 and 10 were loaded with 25, 50 and 100 μg of ER vesicles.

vesicles not retained by magnetic field (lane 7), nor in controls for ER membranes (Fig. 4: lane 8, 25 μg, lane 9, 50 μg and lane 10, 100 μg). The purification of Golgi vesicles was further confirmed using the mouse anti-p28 antibody to detect p28, a specific probe for Golgi. This protein was visualized in the untreated Golgi vesicles (Fig. 4, lanes 1, 2, 3), in Golgi vesicles treated with the pre-immune serum and in the *N*-HSST immunopurified Golgi fraction (lanes 5 and 6), respectively.

To detect the ER contamination of the Golgi vesicles, we used an anti-calreticulin antibody. Fig. 4, lane 1 shows the presence of calreticulin in the untreated Golgi vesicles and in ER vesicles, lanes 8, 9 and 10.

4. Discussion

Subcellular fractionation and purification of organelles has always been a serious challenge in cell biology. Since most of the fractionation protocols make use of physical properties of intracellular membranes such as their density, it is very difficult to prepare highly purified organelles by density-gradient ultracentrifugation of microsomal fractions.

In view of this experimental fact, we designed a procedure to purify Golgi vesicles devoid of ER contamination, based on the binding of an antibody specific for an integral Golgi protein. We selected a protein specific for Golgi, and we developed an antibody to bind the Golgi vesicles. With the help of a secondary antibody linked to magnetic beads, the whole complex could be isolated by means of a magnet. The advantage of this method is that the purified vesicles maintain their physical integrity and can be used for physiological determinations in contrast with other purification methods. Flow cytometry, for example, has been used to purify cells and nuclei; later on, it has been optimized to purify organelles. However, the purification of vesicles by this method brings some difficulties, such as changes in size distributions of vesicles and their physical integrity are not often well resolved. The size of vesicles in a flow cytometer depends on pressure, linear velocity and position of the stream (Bock et al., 1997).

The method developed in this report seems appropriate, since the peptide design and the immunization protocol produced high specific sera. The antibody, characterized by immunofluorescence, showed that HSST was confined to the perinuclear structures typical of Golgi complex. Moreover, immunoelectron microscopy demonstrated that the anti-HSST antibody gold particles bound to Golgi membrane vesicles are included in clear or electron-dense vesicles. Furthermore, the material obtained by immunopurification using magnetic beads showed the presence of HSST and p28 proteins, indicating that this material constitutes a macromolecular complex, which may well be intact Golgi vesicles. This result confirms that the purified material has more components than the HSST protein. In fact, it has been recently demonstrated that

complex microbead-loaded compartments can be selectively separated from the rest of the cell. Perrin-Cocon et al. (1999) were able to isolate functional endocytic compartments involved in antigen processing based on magnetic sorting. This demonstrates that the integrity of macromolecular complex is preserved by magnetic sorting.

In conclusion, we present a simple and reliable method to purify cell organelles based on biological properties rather than physical ones such as ultracentrifugation. This method is based on the use of a highly specific antibody, therefore, different antibodies could be used including some commercially produced. Our interest was to improve the purity of the Golgi vesicles while maintaining their integrity to perform membrane transport experiments, along with their biochemical characterization. We are certain that this method of purification will help to obtain Golgi vesicles free of ER contamination.

Acknowledgements

We thank Dr. María Rosa Bono for the useful discussion and revision of the manuscript. We also thank Dr. Tulio Nuñez for providing us the anti-calreticulin antibody. This work was supported by FONDECYT Grant No. 1970467 to D.W.

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