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Partial purification and MALDI-TOF MS analysis of UN1, a tumor antigen membrane glycoprotein☆

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Abstract

UN1 is a membrane glycoprotein that is expressed in immature human thymocytes, a subpopulation of peripheral T lymphocytes, the HPB acute lymphoblastic leukemia (ALL) T-cell line and fetal thymus. We previously reported the isolation of a monoclonal antibody (UN1 mAb) recognizing the UN1 protein that was classified as "unclustered" at the 5th and 6th International Workshop and Conference on Human Leukocyte Differentiation Antigens. UN1 was highly expressed in breast cancer tissues and was undetected in non-proliferative lesions and in normal breast tissues, indicating a role for UN1 in the development of a tumorigenic phenotype of breast cancer cells. In this study, we report a partial purification of the UN1 protein from HPB-ALL T cells by anion-exchange chromatography followed by immunoprecipitation with the UN1 mAb and MALDI-TOF MS analysis. This analysis should assist in identifying the amino acid sequence of UN1.

Keywords: Membrane glycoproteins; Breast cancer; Protein purification

1. Introduction

The murine monoclonal antibody (mAb) UN1 was previously selected for the specific reactivity with human thymocytes as compared to peripheral blood cells [1]. The antigen recognised by UN1 mAb is a 100–120 kDa transmembrane glycoprotein showing biochemical features of cell membrane-associated mucin-like glycoproteins, a class of macromolecules that are involved in cell-to-cell interactions and cancer progression [2,3]. The UN1 protein was expressed at high levels on the majority of human thymocytes and a small subpopulation of peripheral blood T lymphocytes [1,3]. UN1 was also expressed on the cell membrane of leukemic T-cell line and solid tumors [4]. UN1 expression was investigated on normal and pathological breast tissues by immunohistochemical and Western blotting analysis [5]. The protein was absent in normal breast and benign non-proliferative lesions, moderately expressed in the majority of tested fibroadenomas, and highly expressed in proliferative lesions, both in situ or infiltrating carcinomas. In particular, metastatic lesions showed the highest levels of UN1 [5]. These results underscore a direct correlation between the expression of UN1 and the development of a tumorigenic phenotype of breast tissue. In this regard, UN1 may function as a specific marker of breast cancer.

This study is aimed at the identification of the amino acid sequence of the UN1 protein; to this end, total proteins extracted from HPB-ALL T cells were subjected to anion-exchange chromatography followed by immunoprecipitation with UN1 mAb. The UN1 immunocomplex was fractionated on SDS-PAGE and stained with coomassie blue. The protein band corresponding to UN1 was in situ digested with trypsin. Tryptic fragments were analysed by matrix-assisted laser desorption ionization-time of

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flight mass spectrometry (MALDI-TOF MS). In addition, we also report a strategy to optimize the purity of the sample for the mass spectrometry analysis by coupling the UN1 mAb-mediated immunoprecipitation with a mimotope-based displacement of the UN1 protein.

2. Experimental procedures

2.1. Monoclonal antibody

The UN1 mAb was produced by the hybridoma technology and selected on the basis of its high reactivity with human immature thymocytes, as previously described [1]. UN1 mAb was collected as hybridoma supernatant or ascites and purified by affinity chromatography on protein A-Sepharose (Amersham Biosciences, Uppsala, Sweden) [6].

2.2. Protein extract

HPB-ALL cells (8×10^9) were resuspended in Phosphate Buffered Saline (PBS) lysis buffer containing the Protease Inhibitor Cocktail Complete^{TM} (Roche, Mannheim, Germany) and 1% Triton X-100 (Sigma, St. Louis, USA). The suspension was placed on ice for 30 min, occasionally mixed, and subjected to three cycles of freezing and thawing. The cell lysate was centrifuged at $15{,}000 \times g$ for 30 min at 4 °C and the supernatant (46 ml, 12.2 mg/ml) was recovered for further purification.

2.3. Western blotting and immune detection

The proteins were separated by 8% SDS-PAGE according to the Laemmli method [7] and electroblotted into nitrocellulose membranes at 20 V for 16 h in 150 mM glycine–20 mM Tris. The membrane was pre-incubated with 5% blocking reagent (BioRad, Hercules, CA, USA) in PBS and then incubated with UN1 mAb (2 μg/ml) in PBS containing 5% blocking reagent for 2 h at room temperature, followed by incubation with sheep anti-mouse IgG horseradish peroxidase conjugated (Amersham Biosciences) in PBS in presence of 5% blocking reagent for 1 h at room temperature. The strips were washed, treated with ECL Western blotting kit (Amersham Biosciences) and exposed to X-ray film (Amersham Biosciences). To quantify the UN1 protein, the film was analysed by scanning densitometry and the specific signal was evaluated as number of pixels/μg of protein.

2.4. Chromatographic techniques

Anion exchange chromatography was performed on columns connected to a FPLC system (Amersham Biosciences). In particular, a DEAE-Sepharose Fast Flow (Amersham Biosciences) column (1.6 cm × 10 cm) was equilibrated in 20 mM Tris/HCl, pH 7.8 containing 0.1% Triton X-100 (buffer A) at a flow rate of 2 ml/min. The total protein extract (560 mg) was applied to the column, which was washed with buffer A; bound proteins were eluted with 300 ml of a 0–500 mM NaCl linear gradient in buffer A. The elution profile was monitored by absorbance at 280 nm. Fractions of 10 ml were collected and analysed for the presence of UN1 by Western blotting (Fig. 1a and b). The positive fractions were pooled together and dialysed against buffer A. The sample (70.3 mg proteins) was then applied to a MonoQ HR 10/10 column (Amersham Biosciences) and equilibrated at 1 ml/min with buffer A. The bound proteins were eluted with 30 ml of a 0–500 mM NaCl gradient in buffer A. Fractions of 1 ml were collected and tested for UN1 by Western blotting. Positive fractions were pooled together and dialysed against PBS buffer containing 0.1% Triton X-100, and concentrated by dialysis against PBS buffer, 0.1% Triton X-100, 50% glycerol.

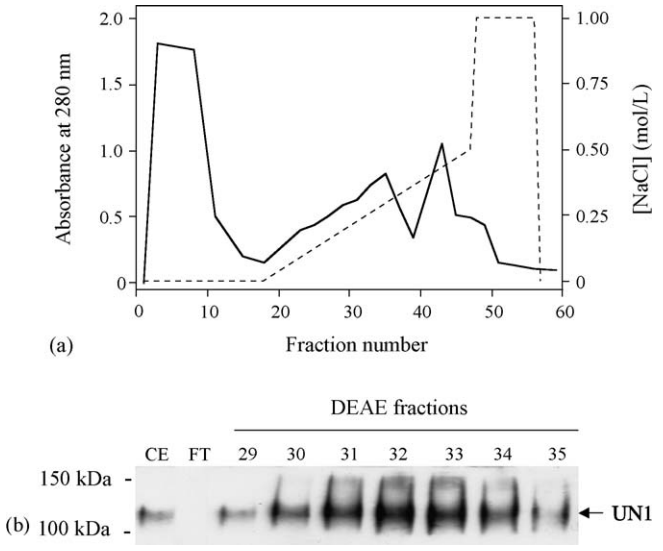

Fig. 1. DEAE-Sepharose Fast Flow chromatography. (a) The total cell-free extract of HPB-ALL was applied on a DEAE-Sepharose column (1.6 cm × 10 cm) equilibrated and eluted as reported in the experimental section. About 10 ml fractions were collected at a flow rate of 2 ml/min. (b) The cell extract (30 μg, first lane), flow through (20 μl, second lane) and eluted fractions (20 μl, following lanes) were separated on 8% SDS-PAGE and analysed by Western blotting using the UN1 mAb.

2.5. Immunoprecipitation

The MonoQ purified sample was ultrafiltrated by Amicon membrane (cutoff > 30 kDa), adjusted to 0.5% Triton X-100 and precleared by incubation with 4 μg of normal mouse IgG (Santa Cruz Biotechnology, CA, USA) coupled to 30 μl of Protein G beads (Amersham Biosciences) in a rotating agitator for 2 h at 4 °C. Following centrifugation at $800 \times g$, the supernatant was incubated with 4 μg of UN1 mAb bound to 30 μl of Protein G beads for 16 h in a rotating agitator at 4 °C. The immunocomplex was recovered by centrifugation and the pellet was washed extensively with PBS, 1% Triton X-100.

2.6. Mimotope-based displacement

The peptide 16-4, which mimics the antigenic epitope of UN1 mAb, was used to elute the UN1 protein from the immunocomplex. The 16-4 mimotope was previously selected by screening a random peptide library displayed on filamentous fd phages using the UN1 mAb [8]. To select the conditions for

the release of UN1 from the immunocomplex, the HPB-ALL cell extract (400 μg) was immunoprecipitated with UN1 mAb (2 μg) bound to Protein G beads (20 μl); then, the immunocomplex was incubated with increasing amounts of 16-4 peptide (0.26, 1.3 or 2.6 mM) in 100 μl of PBS, 0.5% Triton X-100 for 16 h at 4 °C. The UN1 protein was recovered by centrifugation at 800 × *g* for 1 min at 4 °C; supernatant and pellets were analysed for the UN1 presence by Western blotting and quantified by scanning densitometry.

2.7. Colloidal coomassie blue staining

The colloidal coomassie blue staining was prepared by resuspending 0.15% (w/v) Coomassie Brilliant Blue G-250 (BioRad) in 2% (v/v) phosphoric acid and 10% (w/v) ammonium sulphate. After electrophoresis, the gels were fixed in 40% methanol, 10% acetic acid for 1 h and proteins were stained with colloidal coomassie blue containing 20% methanol for 36 h, and destained in water; the limit of protein detection by this method is 10–15 ng.

2.8. In situ digestion

Protein bands were excised from the gel, cut in pieces and washed with acetonitrile and with 0.1 M ammonium bicarbonate. Protein samples were treated with 10 mM DTT for 45 min at 56 °C and alkylated with 55 mM iodoacetamide in 0.1 M ammonium bicarbonate for 30 min at room temperature in the dark, as previously described [9]. Enzymatic digestions were carried out with modified trypsin (Roche) (12.5 ng/μl) in 50 mM ammonium bicarbonate, pH 8.5, at 4 °C for 45 min and then incubated at 37 °C for 18 h. Peptides were extracted by washing the gel with acetonitrile and lyophilised.

2.9. Peptide mapping

MALDI spectra were recorded using a reflectron Voyager DE PRO MALDI-TOF mass spectrometer (Applied Biosystems, Norwalk, CT, USA). An aliquot of the peptide mixture (1 μl) was added to 1 μl of MALDI matrix solution [α-cyano-4-hydroxycinnamic acid (Sigma) 10 mg/ml in acetonitrile: 0.2% TFA = 7:3 (v/v)] and then spotted onto a stainless steel sample plate and allowed to dry. Spectra were acquired in a reflectron mode; the mass range was calibrated using the MH^+ ions from the standard peptide mixture provided by the manufacturers. Raw data were analysed using computer software provided by the manufacturers.

2.10. Database searching

MALDI raw data were inserted into the web-available search program Mascot (http://www.matrixscience.com) to search for non-redundant protein sequence database (NCBInr) by taking advantage of the specificity of the proteolytic enzyme used for the hydrolysis and the taxonomic category of the sample. Proteins were identified by selecting the following item: *Homo sapiens* as taxonomic category; trypsin as enzyme; up to two missed cleavages; cysteines as S-carbamidomethylcysteines; unmodified N- and C-terminal ends; methionines both as unmodified and as oxidised. Only experimental values within 0.03% accuracy were recorded. Three independent sets of experiments were performed.

2.11. Construction of p3xFLAG-CMV-ELKS-α and transfection of HeLa cells

The sequence of ELKS-α cDNA was amplified by PCR from pDR2ELKS-α with the primers 5′-GGA ATT CCA TGT ATG GAA GTG CCC GCT C and 3′-TCC CCC GGG CTA TGC CCA TAT ACC CTC CTC. The PCR product was ligated to p3xFLAG-CMV 7.1 (Sigma) after digestion with *Eco*RI and *Sma*I, to generate p3xFLAG-CMV-ELKS-α. HeLa cells were transfected with 10 μg of p3xFLAG-CMV-ELKS-α using Calcium Phosphate Transfection kit (Invitrogen, Carlsbad, CA, USA) and total protein extract was analysed for the expression of ELKS-α by Western blotting with anti-FLAG M2 mAb (Sigma).

2.12. Protein assay

The concentration of proteins was determined by the method of Bradford [10] using human serum albumin as standard.

3. Results

The HPB-ALL T cells expressing high levels of the UN1 protein were used as source for the purification of UN1. Total protein extract was separated by anion-exchange chromatography using DEAE and MonoQ columns. UN1 was undetected in the flow through fraction of both columns and eluted with NaCl gradient (Fig. 1a). Table 1 summarizes the purification procedure giving a 18% final yield with a purification factor of 8.4. The MonoQ purified sample was immunoprecipitated with the UN1 mAb. The UN1 immunocomplex was separated on SDS-PAGE and stained with coomassie blue; as control, a small fraction of the immunoprecipitated sample was separated on the same gel and

Table 1
Purification steps of UN1

Step	Total protein (mg)	Total activity (pixel)	Specific activity (pixel/mg)	Purification factor	Yield (%)
Cell extract	562.1	432740	769.9	1	100
DEAE	70.3	325677	4632.7	6.0	75
MonoQ	11.8	76960	6522.0	8.5	18

Table 2
Description of the putative candidate ELKS-α

Name	ELKS isoform alpha [*Homo sapiens*]
NCBI (accession no.)	14149661
MW (Da)	108840
MASCOT score	86
Sequence coverage	7%
Number of identified peptides	7

transferred to nylon membrane for Western blotting analysis. UN1 was detected as a 100–120 kDa protein band by Western blotting analysis and undetected by coomassie staining. Next, a band was excised from the gel at the size level of UN1 as detected by Western blotting, and in situ digested with trypsin. The eluted peptides were extracted from the gel for further analysis by MALDI-TOF MS. Maldi spectra were characterised by the presence of several mass signals originated by contaminant keratins. Few signals could be selected for protein identification and used within Mascot software to screen a non-redundant protein data bank. The results led to the identification of ELKS-α [11] as a putative candidate protein with a molecular mass of about 110 kDa (Tables 2 and 3). The identification score was just above the statistical limit thus making necessary a validation of the identification with an independent procedure. In order to verify the candidate gene, the cDNA coding ELKS-α was cloned into the p3xFLAG-CMV eukaryotic expression vector and transiently transfected in HeLa cells. The total extracts from transfected and untransfected cells were analysed by Western blotting using the UN1 mAb and anti-FLAG mAb. The results indicated that UN1 did not correspond to ELKS-α, as the UN1 mAb did not detect the ELKS-α transfected protein (Fig. 2, lanes 1 and 3).

In order to improve the purity of UN1 for the mass spectrometry, the 16-4 peptide mimicking the UN1 epitope recognised by the UN1 mAb was used to remove UN1 from the immunocomplex. To this end, the UN1 immunocomplex was incubated with three dilutions of the 16-4 peptide overnight at 4 °C. The bound (pellet) and the unbound (supernatant) UN1 protein were analysed by Western blotting (Fig. 3a). At the 5000-fold peptide molar concentration, UN1 was displaced from the immunocomplex with a yield of about 75% (Fig. 3b). We plan to scale up this method to allow a liquid proteolytic digestion of UN1 followed by Liquid Chromatography/Mass Spectrometry (LC/MS).

Table 3
Identified peptides by MALDI-TOF MS

Peptide sequence	Position along ELKS isoform alpha sequence	*m/z*	Note
QELESMK	818–824	847.42	*Pyro-glu*(N – *termQ*)
AHEAALEAR	692–700	967.45	
QVKDQNKK	761–768	970.45	*Pyro-glu*(N – *termQ*)
STMTLGRSGGR	87–97	1122.52	
ATEEDHERTR	325–334	1243.61	
ETHLTNLRAER	838–848	1339.61	
DRLVQQLKQQTQNR	898–911	1754.96	

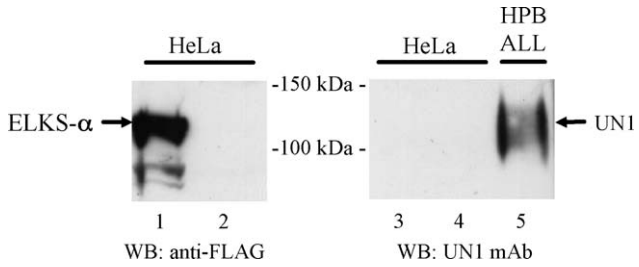

Fig. 2. The UN1 mAb does not recognize the ELKS-α protein. Total extracts (30 μg) of HPB-ALL (lane 5) and HeLa cells transfected with p3xFLAG-CMV-ELKS-α (lanes 1, 3) or untransfected (lanes 2, 4) were analysed by Western blotting using the UN1 mAb or anti-FLAG mAb.

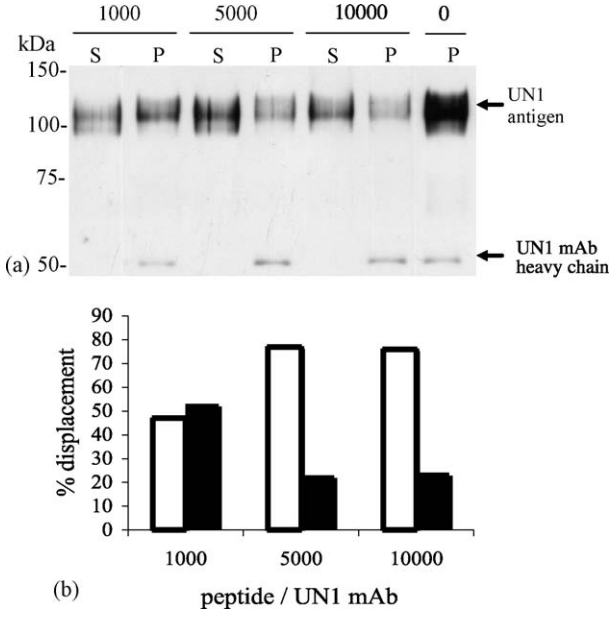

Fig. 3. The 16-4 peptide displaces UN1 from the immunocomplex. (a) The UN1 mAb–UN1 antigen complex was incubated with the indicated 16-4 peptide/UN1 mAb molar ratio. After centrifugation, the supernatant (S) and the pellet (P) were analysed by Western blotting with the UN1 mAb. (b) The displaced (empty bar) and undisplaced (filled bar) UN1, shown in (a), were quantified by scanning densitometry analysis.

4. Discussion

The UN1 mAb detects a membrane glycoprotein with an apparent weight of 100–120 kDa. This antigen appears to be expressed in early stages of T-cell ontogeny (immature thymocytes) [4], a small subpopulation of peripheral blood T lymphocytes, leukemic T-cell line and solid tumors. This paper describes the purification of UN1 protein from HPB-ALL cells using the combination of DEAE, MonoQ ion-exchange chromatography and immunoprecipitation with the UN1 mAb. The immunoprecipitated UN1 was not detected by coomassie blue staining of the gel after SDS-PAGE; this suggests that the amount of purified UN1 was lower then 15 ng, which is considered the limit of protein detection using the Colloidale Coomassie Blue staining.

The MALDI-TOF analysis of the tryptic peptides obtained by in situ digestion of the gel band corresponding to UN1 protein as detected by Western blotting did not provide the identity of UN1 protein. This was probably due to the low amount of purified protein and/or low efficiency elution of UN1 peptides from the gel. Indeed, the size, hydrophobicity, and glycosylation of UN1 could represent an obstacle to the in situ digestion of UN1 on gel. High-sequence coverage is difficult to obtain because the membrane-spanning segments are either not readily accessible to proteolytic enzymes, or lack the specific proteolytic cleavage sites [12,13]. To overcome these hurdles, we are now attempting to purify UN1 from the immunocomplex by using the 16-4 peptide, a mimotope of UN1 antigen. Competition experiments, ELISA and cytofluorimetric analysis have shown that the 16-4 peptide efficiently displaced UN1 from the binding to UN1 mAb. The displaced UN1 will be further analysed by in liquid proteolytic digestion and LC/MS.

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