Identification and Characterization of *Sclerotium rolfsii* Lectin (SRL) Binding Proteins from Human Colon Epithelial Cancer HT29 Cells

Srikanth B[1],
Ravindra G[2], Sachin ME[1],
Prajna H[1], Lu GY[3], Bale MS[1]
and Shashikala RI[1,3]*

1 Department of Studies in Biochemistry, Karnatak University, Dharwad-580 003, India
2 Agilent Technologies India Pvt. Ltd, Bangalore -560048, India
3 Department of Gastroenterology, Institute of Translational Medicine, University of Liverpool, Liverpool, L69 3BX, England

Corresponding author:
Dr. Shashikala R Inamdar

✉ srinamdar2009@gmail.com

Professor of Biochemistry, Post Graduate Dept. of Studies in Biochemistry, Karnatak University, Dharwad-580 003, India,

Tel: +918362771973

Fax: +91836274884

Abstract

Background: TF antigen specific *Sclerotium rolfsii* lectin (SRL) inhibits human colon epithelial cancer HT29 cell growth by induction of apoptosis through cell surface binding and has tumor suppressing effect *in vivo* as reported earlier. Here we report the purification, identification and characterization of SRL binding membrane proteins from HT29 cells.

Methods and Findings: Membrane proteins from HT29 cells were isolated by phase separation and purified by affinity chromatography using SRL-Sepharose-4B matrix. Affinity purified proteins were subjected to in-gel and in-solution trypsin digestion, analysed by ESI-Q-TOF LC-MS and spectrum mill software. Considering the specificity of SRL towards O-glycans, the presence of O-GalNAc sites in SRL interacting proteins were tested using NetOGlyc software. Western blotting was performed to validate the MS identified proteins. A major protein band around 25kDa following in-gel trypsin digestion was identified as Keratin 1 by MS. In-solution trypsin digestion followed by MS identified 25 SRL interacting proteins namely, keratins, heat shock proteins, tubulins, pyruvate kinase M1/M2, peroxiredoxin-1, ATP synthase subunit alpha, mitochondrial, retinal dehydrogenase 1, actin, annexin-A2, prohibitin, ADP/ATP translocase-2 and alpha enolase. NetOGlyc software analysis revealed 21 proteins positive for O-glycosylation sites including keratins alone containing 27 to 50 O-GalNAc sites. Keratin 1 identified and validated by western blotting as major SRL interacting protein showed 49 O-GalNAc sites.

Conclusion: SRL binding membrane proteins from human colon epithelial cancer HT29 cells have been identified and characterized. Identified proteins contain O-GalNAc sites and are known to be involved in cell survival, apoptosis and tumorigenesis. The present study provides insights in studying the mechanism of SRL induced apoptosis and to explore lectin for its clinical implications.

Key words: *Sclerotium rolfsii* lectin; HT29 cell membrane proteins; NetOGlyc version 4.0; Q-TOF-LC/MS; Spectrum Mill.

Abbreviations: SRL: *Sclerotium rolfsii* lectin; LC/MS: Liquid chromatography/Mass spectrometry; ESI: Electro Spray Ionization; Q-TOF: Quadrupole- Time of Flight; PTM: Post Translational Modification; ACN: Acetonitrile; CBB: Coomassie Brilliant Blue; BSA: Bovine Serum Albumin.

Introduction

Lectins are carbohydrate-binding proteins of non-immune origin possessing at least one non-catalytic domain that binds reversibly to specific mono- or oligosaccharides [1]. Lectins are known for their affinities to carbohydrates and hence have been used to detect sugar moieties on normal and transformed cell surfaces and to study the structural and functional role of cell

surface glycans/receptors [2,3]. Lectins are also valuable tools in understanding various physiological processes like cell adhesion and localization, signal transduction across membranes, mitogenic stimulation, augmentation of host immune defence, cytotoxicity and apoptosis [4]. Some plant and fungal lectins are shown to elicit diverse physiological responses such as mitogenic, anti-proliferative, anti-tumour, antifungal and immunomodulatory activities that are generally initiated by recognizing and binding to membrane proteins [5,6]. Some lectins such as Peanut agglutinin (PNA), *Amaranthus caudatus* agglutinin (ACA), *Agaricus bisporous* lectin (ABL), Jacalin (JAC), *Agrocybe aegerita* lectin (AAL) and SRL are known to recognize cancer associated Thomsen-Friedenreich antigen (Galβ1–3GalNAc-α-O-Ser/Thr, T or TF) and their derivatives and are known for exerting their growth stimulatory or inhibitory or apoptosis inducing effect on these cancer cells [7]. Hence identification of specific target cell membrane proteins of TF binding lectins unravels the initial event in the primary action of these lectins on cancer cells and consequent activation of the signalling pathways upon lectin binding [8,9]. Lectin affinity chromatography in combination with mass spectrometry is a powerful tool to identify the membrane proteins recognised by lectins [10]. Identification of lectin interacting proteins is of great significance as it further strengthens clinical application of these lectins in cancer therapeutics.

Our earlier studies have shown that antitumor lectin from *Sclerotium rolfsii* (SRL) is involved in development and morphogenesis of fungus [11,12]. Crystal structure of SRL has been determined in its free form at 1.1 A˚ resolution [13]. Glycan array analysis has revealed the exquisite binding specificity of *Sclerotium rolfsii* lectin towards TF antigen and its derivatives [14]. Recent studies have shown that SRL inhibits cancer cell growth and induces apoptosis in human colon epithelial cancer HT29, ovarian cancer PA1 and breast cancer MCF7 cells by involving both intrinsic and extrinsic pathways as a consequence of its cell surface binding [15-17]. Antitumor effect of SRL has been demonstrated in NOD-SCID mice bearing HT29 xenografts *in vivo* [15]. Gene expression profiling studies in HT29 cells following SRL interaction has revealed that lectin induced differential expression of genes involved in multiple signalling pathways like mitogen activated protein kinase (MAPK), insulin, cell cycle, DNA replication and apoptosis pathways [18].

In the current study, we report the identification of SRL interacting membrane proteins from human colon epithelial cancer HT29 cells. The strategy of protein identification involved isolating SRL binding proteins from membrane enriched fraction by lectin affinity chromatography, followed by proteomics workflow approach using LCMS. The presence of O-GalNAc sites in SRL interacting proteins were predicted by NetOGlyc software and keratins identified as SRL binding proteins were validated by western blotting.

Methods

Cell culture

HT29, human epithelial colorectal adenocarcinoma cells were obtained from the European Cell Culture Collection via the Public Health Laboratory Service (Porton Down, Wiltshire, UK).

HT29 cells were cultured and maintained in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS (Fetal Bovine Serum), 100 units/ml penicillin and 100 μg/ml streptomycin (Complete DMEM) at 37°C in 5% CO_2.

Purification of SRL

SRL was purified from sclerotial bodies as mentioned earlier [11]. Briefly, SRL was extracted from sclerotial bodies of the fungus with 50 mM acetate buffer (pH 4.3) containing 100 mM NaCl and subjected to 30% methanol precipitation and then passed through ion exchange chromatography on CM cellulose column equilibrated with 50 mM acetate buffer(pH 4.3) containing 100 mM NaCl. The bound proteins were eluted with salt gradient from 100 to 600 mM of NaCl in the equilibration buffer. The protein peak fractions with hemagglutinating activity were pooled and dialyzed against 25 mM ethylene diamine-acetate buffer (pH 4.5) and finally the lectin was purified on Superdex G-75 gel filtration column equilibrated with 25 mM Tris-HCl buffer saline, pH 7.5.

Activation of Sepharose-4B and coupling of SRL

SRL coupled Sepharose-4B required for affinity pull down of lectin binding proteins was prepared by CNBr activation of Sepharose-4B and coupling with SRL according to the method described by March et al. [19]. Briefly, Sepharose-4B was suspended in 2 M sodium carbonate (1:1) for the activation and CNBr (0.05 volume) in acetonitrile was added to the Sepharose-4B with constant stirring at room temperature (RT). The slurry was extensively washed with the 0.1 M carbonate buffer (pH 9.5) and resuspended in the binding buffer (0.2 M carbonate buffer, pH 9.5). SRL (10 mg/ml) in binding buffer was added to the slurry and kept for coupling at 4°C for 20 h with constant shaking. Unreacted groups were masked by incubating the slurry with 1 M glycine for 4 h. After coupling the slurry was extensively washed with 0.1 M acetate buffer (pH 4.5), 2 M urea and 0.1 M carbonate buffer (pH 10) containing 0.5 M sodium chloride. SRL coupled Sepharose-4B was suspended in 25 mM TBS pH 7.5 and stored at 4° C until further use.

Membrane protein isolation

HT29 cells were grown in T75 flask in complete DMEM media until they reach the 85-90% confluence. 50× 10^6 cells were harvested by gentle trypsinization, washed with PBS and used for membrane protein isolation by "Mem per isolation kit" (Pierce, USA) as per the manufacturer's instruction. Briefly, for 50×10^6 cell pellet, 1500 μl of Reagent A and 50 μl of PIC (Protease inhibitor complex, Calbiochem, USA) was added, mixed properly and incubated for 10 min at RT with occasional stirring. Lysed cells were placed on ice and 4500 μl of B+C (B:C=1:2) solution was added, vortexed and incubated on ice both for 30 min (vortexed at every 5 min). Samples centrifuged at 9000 rpm for 3 min at 4°C and resulting supernatant was incubated at 37°C for 20 min on water bath for phase separation. Samples centrifuged at 10000 rpm for 2 min at RT to isolate hydrophobic fraction containing membrane proteins. Top layer containing hydrophilic phase was removed and the hydrophobic phase containing membrane protein was stored at -80°C prior to lectin affinity.

SRL-affinity chromatography

SRL-Sepharose 4B and lectin affinity matrix was equilibrated with equilibration buffer TBS (25 mM Tris, pH 7.5, 0.15 M NaCl). The total membrane protein (2 mg) isolated from the HT29 cells was first applied to Sepharose-4B matrix (5 ml) and incubated overnight at 4°C on rocking, to exclude the proteins binding nonspecifically to the resin. Unbound proteins from matrix were collected by spinning and washing with the TBS. The protein fraction was then applied to lectin affinity matrix (5 ml) in a micro centrifuge tube pre-incubated with 3% BSA (Sigma Aldrich, USA) to block non-specific binding sites of affinity matrix and incubated for 12-14 h at 4°C on a rocking platform. Lectin-matrix column was extensively washed with the TBS to remove unbound proteins. After washing, SRL captured proteins were released by treating with elution buffer (25 mM Tris, pH 7.5, containing 0.15 M NaCl and 5% SDS) for 12-14 h at 4°C and eluted fraction was collected by centrifuging the tubes at 3000 rpm for 5 min at 4°C and stored at -80°C. Protein concentration was determined by using protein estimation kit (Bio-Rad, Hercules, CA).

SDS-PAGE and trypsin digestion of membrane proteins

Lectin affinity purified membrane proteins from the HT29 cells were separated by SDS-PAGE on 10% (w/v) acrylamide gels of 0.75 mm thickness. Protein bands were visualized using Coomassie brilliant blue G-250 (Sigma Chemical Co., USA) and the sizes of the resolved proteins were compared with standard molecular weight markers (Merck, India).

Preparation of samples for mass spectrometry analysis: For mass spectrometry analysis, affinity purified membrane proteins were subjected to Agilent's in-gel and in-solution trypsin digestion protocol. Briefly, for in-gel digestion, each band was excised from the gel and destained using acetonitrile (ACN) and ammonium bicarbonate (Sigma Aldrich, USA) solution mixture (80 mg of Ammonium bicarbonate in 40 ml of 50% acetonitrile). Gel pieces were reduced with 50 mM dithiothreitol (DTT) (Sigma Aldrich, USA) for 20 min at 60°C and alkylated with 20 mM iodoacetamide (IAA) (Sigma Aldrich, USA) for 60 min at RT in dark. Gel pieces were dehydrated by soaking in 100% ACN for 5 min, dried using Speed Vac (Eppendorf) concentrator and rehydrated by adding trypsin solution (mass spectrometry grade, 1 μg of trypsin/20 μg of protein) (Promega, Madison, USA) and incubated overnight at 37°C. Supernatant was removed and the gels were extracted for peptides in 1% formic acid (Sigma Aldrich, USA). Both supernatants containing peptides were mixed and concentrated. Peptides were redissolved in water containing 3% acetonitrile and 0.1% Formic acid and analyzed on LCMS system.

For in-solution digestion, 50 μg of SRL purified membrane proteins were dissolved in 25 μl 2,2,2-trifluoroethanol (TFE) (Sigma Aldrich, USA) and 25 μl of ammonium bicarbonate (100 mM) and reduced with 2 ul of DTT (200 mM) for 60 min at 37°C and alkylated with 2 μl of IAA (200 mM) for 60 min at RT in dark. Sample was diluted by ammonium bicarbonate and digested using mass spectrometry grade trypsin solution (1:20) for overnight at 37°C. Trypsinization was inhibited by adding 1 μl of formic acid. Sample was concentrated, redissolved in water containing 3% acetonitrile and 0.1% Formic acid and analyzed on LC/MS system.

HPLC, mass spectrometry and MS/MS identification

The peptides from in-gel or in-solution digestion (5 μl) was applied to Agilent Polaris-HR-Chip-3C18 with a 360 nL enrichment column and 150 mm × 0.075 mm analytical column coupled with the Agilent 6550 iFunnel Q-TOF LC/MS System. The solvent system consisting of 0.1% formic acid in water (A); 90% acetonitrile in water with 0.1% formic acid (B) was used for the analysis. Spectra were recorded in positive ion mode. Data were acquired at 2 GHz (extended dynamic) and Agilent Spectrum Mill MS Proteomics Workbench was used for database search to identify SRL binding membrane proteins.

Prediction of O-GalNAc sites in MS identified membrane proteins

O-GalNAc sites in MS identified SRL binding membrane proteins were predicted by using NetOGlyc version 4.0 software [20]. NetOGlyc version 4.0 is online software used to predict O-GalNAc glycosylation sites in the given protein sequence. SRL binding protein sequences were fetched from Uniprot database. FASTA sequence of these proteins were uploaded in NetOGlyc version 4.0 software and processed to predict the potential O-glycosylation sites [20].

Western blotting

SRL-affinity purified and total membrane proteins were electrophoresed on 10% SDS–polyacrylamide gels, and then blotted on to PVDF (Polyvinylidene difluoride) membrane (Millipore, USA) by wet method. Blots were blocked with 5% BSA, and then probed with Keratin 1 (Abcam, MA, USA), Keratin 8 and Keratin 18 (Sigma Aldrich, USA) mouse primary monoclonal antibodies (1:500) for overnight at 4°C. Blots were washed thrice with TBST and incubated with species specific HRP conjugated secondary antibody (Bio-Rad Laboratories, USA) (1:1000) for 2 h. Blots were washed thrice with TBST and bands were visualized by using chemiluminescence with Immun-Star™ HRP Luminol/Enhancer (Bio-Rad Laboratories, USA). The membranes were exposed to X-ray films, fixed and developed.

Results

Isolation of membrane proteins and purification by using lectin affinity matrix

2 mg of membrane proteins was isolated from $50×10^6$ HT29 cells by phase separation using "Mem per isolation kit" and treated with immobilized lectin matrix. Affinity chromatography of SRL-binding membrane proteins yielded 340 μg of proteins upon elution.

Protein digestion

SRL purified HT29 membrane proteins for the mass spectrometry identification were prepared by both in-gel and in-solution tryptic digestion. In first method, SRL purified proteins were fractionated on the 10% SDS-PAGE (**Figure 1A**) and visual bands were processed

for in-gel tryptic digestion and in second method, affinity eluted sample in-solution form was directly used for trypsin digestion.

In-gel tryptic digestion and Q-TOFLC/MS identification

SRL-affinity purified membrane proteins were resolved on the SDS-PAGE and stained with CBB. SDS-PAGE revealed 6 protein bands corresponding to approximate molecular weights of 80kDa, 66kDa, two bands near 50kDa, 25kDa and 17kDa (**Figure 1A**). The protein band near 25kDa was intense compared to other three protein bands pulled down by lectin affinity.

These protein bands were subjected for in-gel tryptic digestion and the tryptic digest was analysed by ESI-Q-TOF-LCMS/MS. **Figure 1B** shows representative MS/MS spectra of keratin peptides. **Figure 2** displays in-gel digestion of band e protein at 25kDa and identification by Agilent's Spectrum Mill analysis. Spectrum Mill search indicated that 14 and 11 peptides were matched with distinct summed MS/MS search score of 204.46 and 158.31 for Keratin-1 and Keratin-1 isoforms (Keratin 1-7 isoforms) respectively. Protein bands b and f were identified as BSA and SRL respectively (Data not shown), BSA (Bovine Serum Albumin) was used for blocking of non-specific sites and probably the coupled SRL was leached out during affinity pull down chromatography. The protein identification from other three minor bands (a, c and d) did not result in any definite identification probably due to their low abundance.

In-solution tryptic digestion and Q-TOF-LC/MS identification

In second method, SRL-affinity purified membrane proteins were directly subjected for in-solution tryptic digestion and the digest was analysed by ESI-Q-TOF-LC/MS. **Figure 3** displays in-solution tryptic digest identified proteins by Agilent's spectrum Mill software and **Figure 4** displays MS/MS spectra of Keratin 1, annexin-A2, Tubulin alpha-1B chain, HSP 90 and actin. Spectrum Mill search from in-solution tryptic digestion method indicated that 22 peptides matched with distinct summed MS/MS search score 389.63 for Keratin-1. In a similar way 24 proteins excluding Keratin1 derived from in-solution, were also analyzed using the same methods used for identification of Keratin 1. The peptide matches and distinct summed MS/MS search scores *etc*. for other proteins are presented in **Figure 3**. A total of 25 SRL interacting proteins were identified namely, keratins, heat shock proteins, ATP synthase subunit alpha, mitochondrial, retinal dehydrogenase 1, actin cytoplasmin-2, tubulins, pyruvate kinase-M1/M2, annexin-A2, peroxiredoxin-1, prohibitin, alpha enolase and ADP/ATP translocase-2. **Table 1** presents SRL binding proteins, their molecular size, biological response and post translational modifications (PTM) as per the literature.

Prediction of O-GalNAc sites

SRL binding proteins were checked for the presence of O-GalNAc sites by employing NetOGlyc version 4.0. Analysis by NetOGlyc version 4.0 revealed the presence of potential O-GalNAc sites in the identified proteins. Out of 25 proteins, 21 were found to be positive and four were negative for O-GalNAc sites. Supporting

information 1 presents prediction of O-GalNAc sites of Keratin-1 by NetOGlyc version 4.0. O-GalNac sites of other identified proteins were analysed in a similar way (data not shown). The number of potential O-GalNAc sites in the SRL interacting proteins is presented in **Table 2**.

Validation of keratins as SRL binding membrane proteins by western blotting

Western blotting studies were carried out to check and validate keratins identified by MS as SRL binding membrane proteins as keratins are considered as a common contaminant in MS analysis. Membrane blots of total membrane proteins and lectin-affinity purified proteins probed with Keratin1, Keratin 8 and Keratin 18 antibodies and the results are presented in **Figure 4**. A prominent Keratin 1 band was observed in SRL eluted and total membrane protein fraction confirming that Keratin 1 is the one of the binding partner of SRL. In contrast Keratin 8 and Keratin 18 were not detected in both the total membrane and SRL eluted fraction.

Discussion

Antitumor effect of *Sclerotium rolfsii* lectin has been demonstrated earlier both by *in vitro* and *in vivo* studies in human colon epithelial cancer HT29 cells and NOD-SCID mice respectively [15], that demands the need to identify lectin interacting membrane proteins from these cells. In the present study, we report the identification of *Sclerotium rolfsii* lectin interacting membrane proteins containing O-GalNAc sites from human epithelial colon cancer HT29 cells. SRL-affinity chromatography was used to capture membrane proteins from HT29 cells and lectin interacting proteins were identified by mass spectrometry. The presence of O-GalNAc sites in the identified proteins was predicted using NetOGlyc version 4.0. MS investigation revealed 25 SRL binding proteins namely, keratins, heat shock proteins, ATP synthase subunit alpha, mitochondrial, retinal dehydrogenase 1, actin cytoplasmin-2, tubulins, pyruvate kinase-M1/M2, annexin-A2, peroxiredoxin-1, prohibitin, alpha enolase and ADP/ATP translocase-2 and some of these are membrane scaffold proteins. The present study is an attempt to identify SRL binding membrane proteins from enriched membrane fraction however we cannot rule out the possibility of copurification of associated membrane scaffold proteins and few cytosolic proteins. Interestingly these identified lectin interacting proteins are known to be involved in physiological process like cell cycle, apoptosis, angiogenesis, stress response, host-virus interaction, DNA synthesis and glycolysis and hence interaction of SRL with these proteins is likely to affect the said cellular processes.

Keratin 1 is identified as one of the SRL binding membrane protein by MS both by in-gel and in-solution tryptic digestion. Since keratins are known to be common contaminants in MS analysis, we repeated the experiment thrice independently under stringent conditions to rule out that Keratin 1 identified as SRL binding protein is not a contaminant but a true SRL interacting protein. Further, the data was validated by western blotting analysis. Interestingly, in contrast to Keratin 1, Keratin 8 and Keratin 18 identified as SRL binding proteins by MS were not detected in western blotting in both total and affinity purified fraction. This could be due to the fact that all the keratins identified by MS are
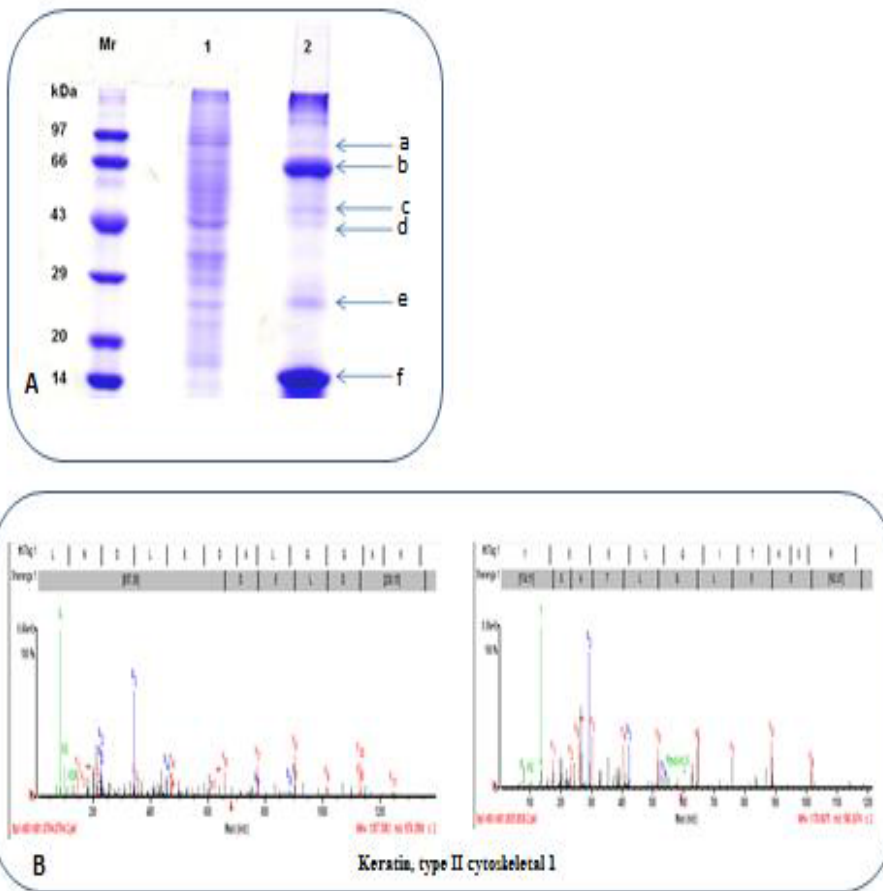
Figure 1 SDS-PAGE and MS MS spectra of peptides from in-gel trypsin digestion. (A) Affinity purified membrane proteins fractionated on 10% SDS PAGE, Mr represents standard molecular weight markers, lane 1 represents total membrane proteins and lane 2 represents SRL eluted proteins, a, b, c, d, e and f. (B) Representative MS/MS spectra of Keratin 1 peptides from Spectrum Mill.

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Database Accession #	Protein Name
1	51	14	204.26	25	8.95e+004	11935049	keratin 1
1	51	14	204.26	25	8.95e+004	119395750	keratin 1
1	51	14	204.26	25	8.95e+004	1346343	Keratin, type II cytoskeletal 1 (Cytokeratin-1) (CK-1) (
1	48	13	192.15	24	9.07e+004	160961491	keratin 1 (epidermolytic hyperkeratosis)
1	42	11	158.31	17	9.49e+004	109096823	PREDICTED: similar to keratin 1 isoform 7
1	42	11	158.31	17	9.49e+004	109096825	PREDICTED: similar to keratin 1 isoform 5
1	42	11	158.31	17	9.49e+004	109096831	PREDICTED: similar to keratin 1 isoform 6
1	42	11	158.31	17	9.49e+004	109096827	PREDICTED: similar to keratin 1 isoform 3
1	42	11	158.31	18	9.49e+004	109096829	PREDICTED: similar to keratin 1 isoform 4
1	42	11	158.31	18	9.49e+004	109096833	PREDICTED: similar to keratin 1 isoform 2
1	42	11	158.31	19	9.49e+004	109096835	PREDICTED: similar to keratin 1 isoform 1

Figure 2 Lectin binding proteins identified from in-gel trypsin digestion. Identification of affinity purified membrane proteins from in-gel tryptic digest of band e of SDS-PAGE by MS/MS and Spectrum Mill.

Group (#)	Subgroup (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Total Protein Spectral Intensity	Database Accession #	Protein Name
1	1.1	70	22	389.63	40.5	9.31e+008	P04264	Keratin, type II cytoskeletal 1
1	1.2	11	6	82.67	16.1	1.18e+007	P05787	Keratin, type II cytoskeletal 8
1	1.3	7	4	71.73	6.7	7.14e+006	P35908	Keratin, type II cytoskeletal 2 epidermal
1	1.4	9	4	70.26	6.5	1.63e+007	P04259	Keratin, type II cytoskeletal 6B
2	2.1	24	8	146.07	16.2	2.44e+008	P13645	Keratin, type I cytoskeletal 10
2	2.2	15	6	102.01	14.1	1.41e+008	P02533	Keratin, type I cytoskeletal 14
3	3.1	25	8	145.81	23.7	1.03e+008	P63261	Actin, cytoplasmic 2
4	4.1	9	6	104.78	11.1	1.10e+007	P08238	Heat shock protein HSP 90-beta
5	5.1	22	5	94.91	8.1	3.50e+008	P35527	Keratin, type I cytoskeletal 9
6	6.1	6	5	79.03	14.4	8.07e+006	P07355	Annexin A2
7	7.1	10	4	78.17	16.5	4.68e+005	P05783	Keratin, type I cytoskeletal 18
8	8.1	4	4	68.53	12.9	1.26e+006	P06733	Alpha-enolase
9	9.1	6	3	55.99	8.3	7.23e+006	P10809	60 kDa heat shock protein, mitochondrial
10	10.1	5	3	52.46	9.9	4.26e+007	Q05639	Elongation factor 1-alpha 2
11	11.1	6	3	49.35	10.6	2.80e+005	P35232	Prohibitin
12	12.1	5	3	48.18	5	6.80e+007	P02768	Serum albumin
13	13.1	4	3	47.47	6.9	2.01e+006	P14618	Pyruvate kinase isozymes M1/M2
14	14.1	7	2	43.86	14.3	2.82e+007	P84077	ADP-ribosylation factor 1
15	15.1	5	2	37.37	40	2.07e+007	P06703	Protein S100-A6
16	16.1	3	2	36.43	4.7	6.79e+005	P68371	Tubulin beta-4B chain
17	17.1	7	2	34.83	3.2	9.08e+006	P13647	Keratin, type I cytoskeletal 5
18	18.1	4	2	32.91	9.5	1.39e+007	Q06830	Peroxiredoxin-1
19	19.1	2	2	32.63	4.5	4.19e+004	P25705	ATP synthase subunit alpha, mitochondrial
20	20.1	3	2	31.87	6.8	1.14e+006	P68363	Tubulin alpha-1B chain
21	21.1	2	2	30.88	4.1	2.60e+005	P00352	Retinal dehydrogenase 1

Figure 3 Lectin binding proteins identified from in-solution trypsin digestion. Affinity purified membrane proteins subjected to in-solution tryptic digestion and identified by MS/MS and Spectrum Mill.
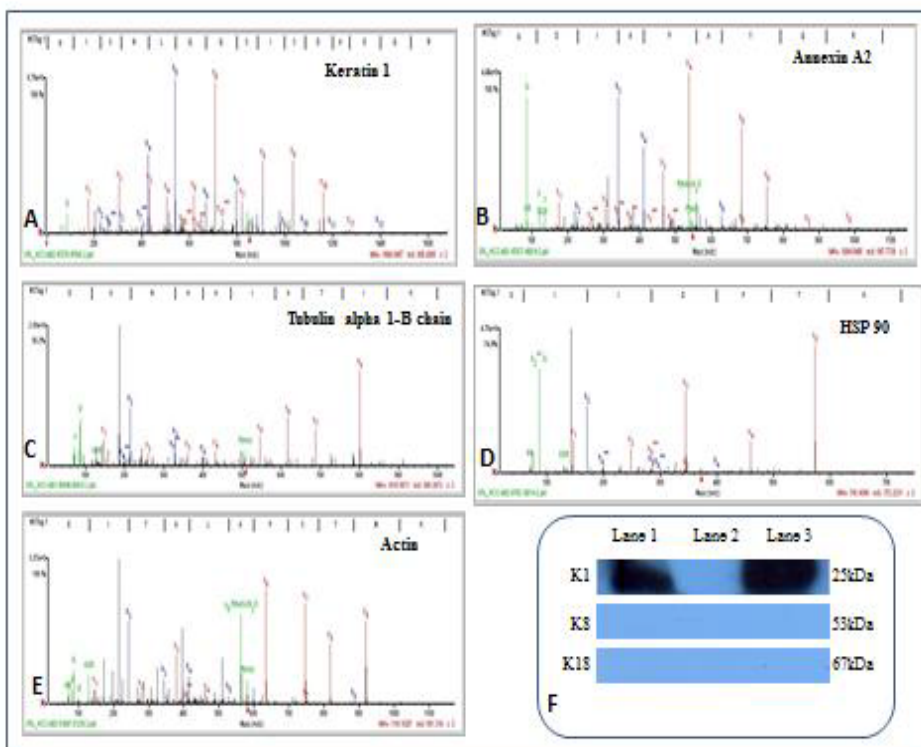

Figure 4 MS MS spectra of peptides from in-solution trypsin digestion and western blotting. Representative MS/MS spectra of (A) Kerain 1, (B) Annexin-A2, (C) Tubulin alpha-1B chain, (D) HSP 90 and (E) Actin peptides. (F) Western blotting; Total membrane protein and SRL-affinity purified proteins from human colon cancer HT29 cells were transblotted to PVDF membrane and probed with Keratin 1 (K1), Keratin 8 (K8) and Keratin 18 (K18) monoclonal antibodies. Data are representatives of two similar experiments.

isoforms which share similar conserved sequences. MS identifies a protein by matching peptide sequences and hence peptides derived from tryptic digestion of Keratin 1 are likely hit others keratins. It is interesting to note that Keratin 1 identified as SRL

Table 1 Proteins identified by MS/MS as SRL interacting proteins, their biological role and PTM.

Serial No.	Identified proteins	Molecular weight[a] (kDa)	Biological response[b]	PTM[c]
1	Keratin 1, type II cytoskeletal 1	65	Complement activation, lectin pathway, Regulation of angiogenesis, Response to Oxidative stress, Fibrinolysis	Citrullination Methylation Phosphoprotein
2	Keratin, type II cytoskeletal 5	58	Cell junction assembly, Epidermis development	-
3	Keratin, type I cytoskeletal 8	60	Host-virus interaction	Acetylation Glycoprotein Phosphoprotein
4	Keratin, type I cytoskeletal 9	62.06	Epidermis and skin development, Spermatogenesis	Phosphoprotein
5	Keratin, type I cytoskeletal 10	58.82	Cellular response to calcium ion, Keratinocyte differentiation	Phosphoprotein
6	Keratin, type II cytoskeletal 2 epidermal	65.43	Associated with keratinocyte activation, proliferation and keratinization	Phosphoprotein
7	Keratin, type II cytoskeletal 6B	60.06	Ectoderm development	Acetylation Phosphoprotein
8	Keratin, type I cytoseletal 14	51.56	Aging, Cell junction assembly, Epithelial cell differentiation, Response to ionizing radiation and zinc ion	Disulfide bond
9	Keratin, type I cytoskeletal 18	67	Cell cycle Host-virus interaction	Acetylation Glycoprotein Phosphoprotein
10	ADP-ribosylation factor 1	20.69	GTPase activity Receptor signalling protein activity	Acetylation Lipoprotein
11	Heat shock protein HSP 90-beta	84	Stress response	Acetylation Glycoprotein Phosphoprotein S-nitrosylation Ubl conjugation
12	Annexin-A2	36	Angiogenesis, Membrane budding, Protein heterotetramerization	Acetylation Phosphoprotein Ubl conjugation
13	Alpha-enolase	47	Glycolysis	Acetylation Phosphoprotein Ubl conjugation
14	60 kDa heat shock protein, mitochondrial	60	Host-virus interaction	Acetylation Phosphoprotein
15	Tubulin beta-4B chain	49.83	'de novo' post-translational protein folding, G2/M transition of mitotic cell cycle	Acetylation Phosphoprotein
16	Tubulin alpha-1B chain	50.12	'de novo' posttranslational protein folding, Cell division	Acetylation Isopeptide bond Methylation Nitration Phosphoprotein Ubl conjugation
17	Peroxiredoxin-1	22	Cell proliferation, hydrogen peroxide catabolic process,	Acetylation Disulfide bond Phosphoprotein

18	Protein S100-A6	10	Signal transduction	Acetylation
19	Elongation factor 1-alpha 2	50.47	Protein biosynthesis	Acetylation Methylation Phosphoprotein
20	Actin, cytoplasmic 2	42	Adherens and Cell-cell junction organization, Cellular component movement	Acetylation, Methylation, Oxidation
21	ADP/ATP translocase 2	32.85	Chromosome partition Host-virus interaction Transport	Acetylation Methylation
22	Prohibitin	29.80	DNA synthesis	Acetylation Phosphoprotein
23	Pyruvate kinase isozymes M1/M2	57.93	Glycolysis	Acetylation Hydroxylation Phosphoprotein Ubl conjugation
24	ATP synthase subunit alpha, mitochondrial	59.75	ATP synthesis Transport	Acetylation Glycoprotein Phosphoprotein
25	Retinal dehydrogenase 1	54.86	Cofactor and Retinol metabolism	Acetylation Phosphoprotein

[a]Molecular size, [b]biological roles and [c]PTM are fetched from Uniprot database.

Table 2 O-GalNAc sites in SRL binding proteins as predicted by NetOGlyc version 4.0.

Serial No.	SRL binding proteins	O-GalNAc sites with *prediction score =>0.5
1	Keratin 1	49
2	Keratin 2	48
3	Keratin 5	50
4	Keratin 6B	34
5	Keratin 8	37
6	Keratin 9	48
7	Keratin 10	34
8	Keratin 14	27
9	Keratin 18	34
10	ATP synthase subunit alpha, mitochondrial	10
11	Retinal dehydrogenase 1	6
12	60 kDa heat shock protein, mitochondrial	5
13	Annexin-A2	4
14	Pyruvate kinase isozymes M1/M2	3
15	Prohibitin	2
16	Heat shock protein HSP 90-beta	2
17	Peroxiredoxin-1	2
18	Actin cytploasmic 2	2
19	ADP/ATP translocase 2	1
20	Tubulin beta-4Bchain	1
21	Elongation factor 1-alpha 2	1
22	ADP-ribosylation factor 1	Nil
23	Protein S100-A6	Nil
24	Alpha-enolase	Nil
25	Tubulin alpha-1B chain	Nil

*Prediction confidence score => **0.5** are forecasted as glycosylated.

binding protein from in-gel digestion of band at approx. 25kDa but the actual molecular weight of Keratin 1 is approx. 65 kDa suggesting that this could be possibly modified keratin expressed either on the cell surface or as an integral membrane protein. There are reports on the expression of modified form of keratins with varying molecular mass that are expressed on cancer cell surface. For example, Borup-Christensen P et al. have shown that the COU-1, a fusion form of antibody recognizes modified forms of keratins with molecular masses of approximately 43 kDa although the native cytokeratin 8 and 18 have molecular masses of 60kDa and 67kDa respectively [21,22]. Further COU-1 antibody has also been used in demonstrating the differential staining of membrane-associated cytokeratins on viable carcinoma cells and resting cells [23]. Taken together collectively, these results suggest that Keratin 1 is one of the major SRL interacting proteins in HT29 cells.

Glycan array analysis of SRL has revealed its specificity towards TF antigen and TF associated O-linked glycans [14], considering this we checked for the presence of O-GalNAc sites in SRL affinity purified proteins by using NetOGlyc version 4.0. This is online software which predicts and records O-GalNAc glycosylation sites in the protein sequence and gives the prediction confidence scores and forecasts sites as glycosylated whose prediction confidence scores are higher than 0.5 and marks them with "#POSITIVE" string [20]. It is interesting to note the data analysed by NetOGlyc version 4.0 software revealed that, out of 25 SRL binding proteins identified by MS, 21 proteins were found to contain potential O-GalNAc sites and only other four proteins, namely alpha enolase, ADP-ribosylation factor 1, protein S100-A6 and tubulin alpha-1B chain were negative for O-glycosylation sites. Since affinity column was blocked with BSA to avoid nonspecific protein-protein interaction, these 4 nonglycosylated proteins captured by affinity column may be through indirect lectin binding (lectin-target glycoprotein-non-target proteins). SRL interacting keratins showed 27 to 50 O-GalNAc sites in their

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sequences. Keratin 1 validated as one of the SRL binding protein contains 49 potential O-GalNAc sites. ATP synthase subunit alpha, mitochondrial, Retinal dehydrogenase 1, HSP-60, annexin-A2 and pyruvate kinase M1/M2 contains 10, 6, 5, 4 and 3 O-GalNAc sites respectively. Other SRL interacting proteins, prohibitin, HSP-90, actin cytoplasmic-2 and peroxiredoxin-1 contain two O-GalNAc sites. ADP/ATP translocase-2, tubulin beta-4B chain and elongation factor 1-alpha-2 contain one O-GalNAc site. Taken together, the analysis suggests that almost all of the SRL-affinity pull down proteins contain potential O-GalNAc sites which further support affinity of SRL towards O-glycans [14].

Lectins due to their sugar recognition property have been valuable tools in identifying glycoconjugates, examples include ConA and WGA. N-glycoproteins from liver cells were identified by using ConA and WGA affinity columns [24] and interestingly few of these identified proteins overlapped with SRL binding proteins. Keratin 1, Keratin 10 and alpha enolase are the common glycoproteins identified by these lectins. Annexin-A2, pyruvate kinase and prohibitin were recognised by WGA and SRL. Keratin 18 was recognised by ConA and SRL. SRL, ConA and WGA also recognised common family of proteins namely tubulins, heat shock proteins and elongation factors. ConA and WGA have specificity for N-glycoproteins and SRL recognizes mostly O-glycoproteins hence these common proteins recognised by these lectins may contain both N and O-glycans. Keratin 1 is identified as one of the SRL interacting protein in HT29 cells. Recognition of keratins by lectins has been reported earlier for example, galactose specific lectins such as recognising keratins, GCA, a galectin from the sponge *Geodia cydonium*, VVA-B4, tetrameric isolectin B4 from *Vicia villosa* and SBA, lectin from *Glycine max* are known to recognize keratins, Keratin 8, Keratin 18, and Keratin 19 from MCF7 cells [25].

Keratins are part of the family of intermediate filament proteins participating in the cytoskeletal assembly of cells. However recent studies have shown that functions of keratins are not restricted as structural proteins but they are also involved in cell signalling, apico-basal polarization, motility, cell size, protein synthesis and membrane trafficking. Keratins are used as diagnostic and prognostic tumor markers and are also recognised as a new class of receptors, for example membrane expression of keratin 1 by HUVEC cells has been shown and it is identified as kininogen receptor [26-28]. These reports support role of keratins as membrane receptors.

Apart from keratins, SRL also recognises other HT29 proteins with O-GalNAc sites namely, prohibitin, annexin-A2, peroxiredoxin 1, pyruvate kinase, heat shock proteins as identified by MS that are linked to apoptosis, cancer cell proliferation and metastasis. Prohibitin (PHB) has been localized to mitochondria, plasma membrane and nucleus and has biological role in mitochondrial function, cell proliferation and development. Phosphorylation of PHB by Akt, TGF-b or Ras ⁄ Raf pathways favours cell proliferation hence inhibition of PHB phosphorylation is hypothesized to inhibit cell proliferation [29]. O-GlcNAc modification and tyrosine phosphorylation of PHB has shown to play a significant role in tyrosine kinase signalling pathways namely, insulin, growth factors and immune receptors signalling [30]. Over expression of

PHB is reported in lung, prostate carcinoma and liver cancer cells and its expression levels is correlated with the proliferation and metastasis of liver cancer cells [31]. Hence integrated targeting of multiple functions of PHB may affect diverse signalling pathways and in turn inhibits cancer cell growth. SRL may be inducing its observed apoptotic effect in HT29 cells by binding to PHB and inhibiting its tumorigenic effect.

Annexin-A2 (Axn A2), another SRL binding protein is localized to the plasma membrane of endothelial cells, tumor cells and regulates diverse cellular functions, including angiogenesis, proliferation, apoptosis, cell migration, invasion and adhesion. Cell surface Anx A2 complexes with plasminogen and tissue plasminogen activator, activates plasmin and thereby is linked to cancer metastasis [32]. Over-expression of Axn A2 has been reported in breast, liver, prostate, gliomas and cancer of pancreas and its expression is correlated with cancer invasion and metastasis. Hence targeting Anx A2 is also considered as a novel therapeutic strategy to inhibit tumor growth [33].

Pyruvate kinase (PK) catalyzes a rate-limiting final reaction in glycolysis, generating pyruvate and ATP. An ample amount of energy is required for survival of cancer cells and uses glycolysis as primary source of energy production also called aerobic glycolysis or the Warburg effect [34] and is regulated by pyruvate kinase M2 (PKM2). Studies have shown that PKM2 is overexpressed in many cancer cells including colon cancer and is correlated with colon cancer proliferation and metastasis. Overexpression of PKM2 also promotes CRC cell migration and cell adhesion by regulating STAT3-associated signalling [35]. In contrast, inhibition/knockdown of PKM2 resulted in the suppression of xenograft tumor growth *in vivo* and thus PKM2 is considered as a potential therapeutic target for CRC [36]. Nonglycolytic role of PKM2 is reported in nucleus of cancer cells, where it activates transcription of many genes and favours cancer growth [37]. Interaction of SRL with PKM2 is likely to block its tumour promoting activity.

Peroxiredoxin-1 (Pxr-1) regulates hydrogen peroxide signalling, acts as a protein chaperone and immune modulator. Overexpression of Prx-1 is reported in many cancers and influences diverse cellular processes including cell survival, proliferation and apoptosis. Pxr-1 overexpression is correlated with poor clinical outcomes and diminished overall patient survival [38,39]. Heat shock proteins (HSPs) are vital molecular players in the cellular stress response. HSPs prevent cell death, for instance by preventing caspase-dependent (e.g., HSP27, HSP70, and HSP90) and/or independent (e.g., HSP70) pathways [40]. However, some HSPs induce cell death, for instance, HSP60/HSP10/pro-caspase complex triggers apoptosis in jurkat cells [41]. Conversely, HSP60 overexpression is reported in various tumors including cervical cancer and is correlated with tumor carcinogenesis [42]. HSP90 regulates and stabilizes deregulated proteins involved in apoptosis, signal-transduction pathways and cell-cycle regulation thereby stabilizes tumorogenic cells. Hence HSP90 is considered as a potential target for drug screening. HSP90 inhibitors such as geldanamycin, 17AAG, 17DMAG, radicicol, oxime derivatives, purine-scaffold inhibitors and novobiocin are used to study their anticancer effect and some of these are in Phase I and Phase II clinical trials [43]. Hence interaction of SRL with HSP90 is likely to block its tumorigenic effect.

Actin has manifold biological functions including cell contraction, motility, vesicle trafficking, intracellular organization, cytokinesis, endocytosis and apoptosis. Actin cytoskeleton is shown to act both as a sensor and mediator of apoptosis [44]. Disruption of actin filament integrity with cytochalasin D and jasplakinolide is shown to induce apoptosis in airway epithelial cells [45]. SRL may be binding to and altering the actin filament integrity in HT29 cells thereby inducing observed HT29 cellular apoptosis. The present study supports the activation of multiple signalling pathways by SRL [18] possibly by binding to more than one membrane proteins and inducing apoptosis in colon cancer HT29 cells.

Conclusion

In conclusion, SRL interacting membrane proteins containing O-GalNAc sites from human colon epithelial cancer HT29 cells have been purified, identified and characterized and interestingly all of them have their reported functions that is concerned with cell survival, angiogenesis or cellular apoptosis. Hence it is likely that SRL induces cellular apoptosis as reported earlier by binding to and inhibiting/altering the function of these identified proteins

implicated in cancer cell progression and metastasis, however it needs further detailed investigation to identify which of these protein initiates cellular apoptosis following its interaction with the lectin. The present study provides insights in studying the mechanism of SRL induced apoptosis and to explore lectin for its clinical implications.

Supporting Information

Supporting information 1 presents prediction of O-GalNAc sites of Keratin-1 by NetOGlyc version 4.0.

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Competing Interests

The authors declare that they have no competing interest.

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