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Modulation of PP2A activity by Jacalin: is it through caveolae and ER chaperones?

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Abstract Plant lectins have been reported to affect the proliferation of different human cancer cell line probably by binding to the specific carbohydrate moieties. In the present study, Badan labeled single cysteine mutant (present in the caveolin-1 binding motif) of jacalin (rJacalin) was found to penetrate the target membrane, indicating a protein-protein or protein-membrane interaction apart from its primary mode of binding *i.e.* protein-carbohydrate interaction. Further, Jacalin treatment has resulted in the movement of the GFP-Caveolin-1 predominantly at the cell-cell contact region with much restricted dynamics. Jacalin treatment has resulted in the perinuclear accumulation of PP2A and dissociation of the PHAP1/PP2A complex. PP2A was found to act as a negative regulator of ERK signaling and a significant decrease in the phosphorylation level of MEK and AKT (T308) in A431. In addition, we have also identified several ER resident proteins including molecular chaperones like ORP150, Hsp70, Grp78, BiP of A431 cells, which were bound to the Jacalin-sepharose column. Among various ER chaperones that were identified, ORP150 was found to present on the cell surface of A431 cells.

Keywords Jacalin · Heat shock proteins · Protein tyrosine phosphatase 2A · AKT dephosphorylation · Caveolae · Kiss and run dynamics

Abbreviations

RT	Room Temperature
IPA	Immunoprecipitation assay
TIRFM	Total Internal Reflection Fluorescence Microscopy

Introduction

Plant lectins are well known unique group of proteins that agglutinate erythrocytes of diverse species [1]. It has been noted that plant lectins also affect the proliferation of various cancer cells probably by binding to specific carbohydrate moieties [2]. For example Peanut agglutinin (PNA) stimulates the proliferation of human intestinal epithelial cells, while *Agaricus bisporu*s lectin (ABL) is a non-cytotoxic inhibitor of proliferation of human colon cancer HT29 cells. In addition, *Vicia faba* agglutinin stimulates differentiation of undifferentiated LS174T human colon cancer cells [3]. Jacalin was found to be non-cytotoxic inhibitor of proliferation of HT29 colon cancer cells, but elicits different responses on other cell types depending on the lineage of the target cell. For example, it was found to be cytotoxic inhibitor of proliferation of human epidermoid carcinoma A431 cells [4]. Jacalin was also shown to down regulate the phosphorylation of ERK in HT29 and A431 cells. While Jacalin was mitogenic to T cells and was shown to upregulate ERK phosphorylation in $CD4^{+}$T cells [5]. Recent reports have shown that Jacalin treatment results in modulation of activity of PP2A (a serine/theronine phosphatase), which has been shown to play an important role in cell survival/death by acting as a negative regulator of ERK1/2 signaling in HT29 cells [6]. In view of the different responses elicited by Jacalin on

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different cells it is important to investigate the mode of binding of jacalin *i.e.* whether or not protein-protein, protein-carbohydrate or protein-membrane interactions responsible for different cellular responses. In addition, the proteins of target cells that bind to Jacalin and the significance of those interactions have also been investigated to identify the proteins of A431 cells that were bound to Jacalin-sepharose column.

Materials and methods

All the reagents used were of analytical grade. Human recombinant TGFα, Methyl-α-Galactoside, were obtained from Sigma chemicals Co. USA. DMEM, Fetal bovine serum was from GibcoBRL, Life tech and chemiluminescence detection kits were purchased from New England Bio-Labs. Recombinant protein-G/A agarose beads were from Invitrogen, Life Technologies. Vector Shield anti-fade mounting medium (Vector Laboratories), protein estimations were carried out by Bradford protein estimation kit from Bio-Rad, USA.

Cell culture A431 and human cervical cancer HeLa cells were cultured in DMEM medium buffered with 2.5 mM *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonate (HEPES) supplemented with 10% FCS and antibiotics (streptomycin sulfate and penicillin G).

Purification of Jacalin Jacalin (*Artocarpus integrifolia*) lectin from jackfruit seed and recombinant Jacalin as described earlier [4].

Cysteine modification by Badan and membrane penetration assessment of rJacalin rJacalin was labeled with Badan at room temperature (RT) for 1 h in the presence of 10 mM DTT. Inactivation of unreacted badan was achieved by addition of 200 mM DTT and removed by passing though desalting column. Labeled (5 μg) rJacalin was added to in 1 ml 0.25% RBCs in absence or presence 100 mM or 200 mM of Methyl-α-D-galactoside for 1 h and badan emission spectra was recorded for 0 min time point and incubated for 1 h. In absence of Methyl-α-D-galactoside total agglutinated rRBCs was excited and emission spectrum was measured after proper mixing of sample. Then the sample incubated with 100 mM of Methyl-α-D-galactoside was briefly centrifuged to separate agglutinated pellets and supernatants. KPBS dissolved pellets and supernatant were exited at 408 nm and emission spectra was recorded between 400–600 nm. Agglutinated rRBCs by Jacalin emission spectra was subtracted all form test spectra.

Transfection of GFP-Caveolin-1 in HeLa For transfection with GFP-Caveolin-1 (gift by Dr. Halineus) HeLa cells were seeded with a density of 2×10^5 cells/ml in 35 mm culture dish, transfection was done at the confluency of 60–70% using fuGENE 6 (roche) with 1 μg of plasmid in a 6:1, complex was replace by complete media 4 h after Transfection.

Total Internal Reflection Fluorescence Microscopy (TIRFM) TIR FM was performed in CO_2-independent medium, 12 h after transfection, the glass coverslip (25 mm thickness) were fixed in Atto chamber (Promega) and DMEM without phenol red. The dynamics of Cav1-GFP in regions of the cell closest to the coverslip were recorded before treatment till 2 h after treatment with Jacalin. The recording was made at 10 Hz at 10 ms exposure time for 200 frames. The images were acquired on Olympus microscope and Argon-Krypton laser (Spectra Physics) at 488 nm was used for excitation with a 100/NA 1.45 oil immersion objective, and using Metamorph vision 4.0 software.

Confocal microscopy A431 cells were cultured on glass cover slips, rinsed with PBS thrice. Cells were then fixed in 3.7% paraformaldehyde in PBS at RT followed by permeabilization with 0.1% TritonX-100 containing 0.5 M NH_4Cl in PBS at RT or without permeabilization. The cells were blocked in 5% BSA in PBS at RT for 30 min. The cells were then incubated with respective antibodies, Caveolin-1(BD Transduction Laboratories - 610406), PP2A (Santa cruz, sc-14020), ORP150 (Abcam, ab56680) for 2 h at room temperature followed by washing with PBS and incubation with appropriate fluorescent secondary antibody for 1 h at room temperature. The cells were then washed four times with PBS and incubated with DAPI for 5 min at RT. The cells were then washed and mounted on the glass slide and were visualized in Zeiss LSM 510 microscope under confocal settings.

Immunoprecipitation A431 cells were seeded at a density of 1×10^6 cells per plate into 60 mm plates and allowed to adhere for 8 h at 37°C. Cells were treated with 40 μg/ ml concentrations of Jacalin incubated for 1 h. After treatment cells were scraped immediately and centrifuged. Cell pellet was lysed in 500 μl of immunoprecipitation assay (IPA) buffer (10 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.05% SDS, 1 mM Na_3VO_4, 1 mM phenyl methyl sulphonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). The cell lysate was centrifuged at 4°C for 10 min at 14,000 rpm, and the supernatant was pre-cleared by incubating with recombinant protein G-agarose beads at 4°C for 6 h, followed by centrifugation at 4°C for 1 min, at 14,000 rpm. The supernatant was subjected to immunoprecipitation by the addition of 2 μg of goat polyclonal anti PHAP1 (santa cruz, sc56520) Antibodies tube was then incubated overnight at 4°C followed by incubation with recombinant protein G

agarose beads at 4°C for 2 h. After washing the beads three times with ice-cold IPA buffer containing the detergent and three times with IPA buffer without detergent were probed with PP2A (santa cruz, sc-14020) and again after stripping the same membrane with PHAP1 Antibodies.

PP2A knockdown by siRNA A431 cells were seeded at the density of 2×10^5 cells/ml in 35 mm plate. For transfection 7.2 µl of 10 µM siRNA was added to 80 µl siRNA transfection medium, mixed gently and kept at room temperature for 5 min (mixture 1) and 4.8 µl of siRNA transfection reagent to 80 µl of siRNA transfection medium, mixed gently and kept at room temperature for 5 min (mixture 2). Mixture 1 and mixture 2 were combined to form siRNA-siRNA transfection reagent complex, mixed gently and kept for 20 min at room temperature. For each transfection 0.64 ml of siRNA transfection medium to each tube containing the siRNA-siRNA transfection reagent complex, mixed gently and overlaid the diluted complex solution on the cells and incubated for 5–7 h at 37°C. The expression PP2A, ERK1/2 was assayed by immunoblot.

Total cellular phosphorylation and phosphorylation of MEK, Raf-1, Akt A431 cells (2.0×10^5) were seeded in six well plates till 70% confluent. Cells were treated with 40 µg/ml of Jacalin for indicated times. The wells were washed with PBS and the cells were scraped from the well, the cell pellet was recovered by centrifugation, resuspended in Lysis buffer containing protease inhibitors (10 µg/ml leupeptin, 2 mM phenylmethylsulfonylfluoride (PMSF) and 2 mM Na_3VO_4. After protein estimation equal protein was loaded on SDS PAGE, the proteins were transferred to a nitrocellulose membrane using tris-glycine buffer pH 8.0 and were probed with anti-phosphotyrosine (PY20) (Santa cruz,sc-508), p-Raf-1(Cell signaling,#9421), p-MEK (Cell signaling #9121 Cell Signaling), MEK, p-Akt1/2/3 (Thr 308,Santa cruz, sc-16646-R), p-Akt1/2/3 (ser 473-R, Santa cruz, sc-7985-R), anti-mouse IgG, HRP-linked Antibodies (cell signaling,#7076), anti-rabbit IgG, HRP-linked Antibody (cell signaling,#7074), HRP detection reagent, Lumi-GLO™ reagent and peroxide (cell signaling #7003).

Isolation of Jacalin bound proteins of A431 cells The Jacalin (1 mg) was coupled with 500 mg of CNBr activated sepharose 4B as per the suggested protocol of the manufacturer. The column was thoroughly washed with 10-bed volumes of PBS (pH 7.4) before use. The matrix and A431 whole cell lysate (7 mg protein) were mixed by rotation for 2 h at 4˚C. The beads were then packed into a column (1×15 cm) and washed thoroughly with PBS till no protein was detected in the wash. Jacalin-sepharose bound proteins were eluted with 50 mM methyl-α-galactoside. Elutes were precipitated with acetone and analyzed.

Result and discussion

Plant lectins affect the proliferation of various mammalian cells probably by binding to specific carbohydrate moieties [2]. For example, Peanut agglutinin (PNA) stimulates the proliferation of human intestinal epithelial cells, while others like *Agaricus bisporu*s lectin (ABL) and Jacalin are non-cytotoxic inhibitors of proliferation of human colon cancer HT29. Interestingly, Jacalin, has the same sugar specificity as PNA towards the human malignancy associated Thomsen-Friedenreich disaccharide (TF disaccharide: Galβ1-3GalNAcα), but elicits different responses depending on the lineage of the target cells [7–9]. It was shown earlier that jacalin, which is a mitogen to T-cells, can also interact with deglycosylated, $CD4^+$ T-lymphocytes through protein-protein interactions [10]. Hence, there is a possibility that the cellular responses mediated by lectins could not be entirely due to lectin-carbohydrate interaction alone but other possibilities such as lectin—target protein interaction may also contribute to the lectin mediated cellular responses.

We have earlier shown that the Jacalin was found to be cytotoxic inhibitor of proliferation of A431 (epidermal carcinoma cell) where, it exerted reversible stress. It induced the phosphorylation of Caveolin-1 and p38 and also decreased the phosphorylation level of EGFR and ERK1/2 in the same cells [4]. In contrast, Jacalin was mitogenic to $CD4^+$ T cells and was shown to up regulate ERK phosphorylation. In order to understand these diverse cellular responses mediated by Jacalin, we have analyzed the primary sequence of Jacalin. A close examination of its primary sequence suggested us that it has a putative Caveolin-1 binding motif (^{120}**Y**-W-L-D-Y-**F**-S-M-**Y** ^{128} resembling the consensus motif ΦXXXXΦXXΦ where Φ is Trp or Tyr or Phe). However, the role played by this Caveolin-1 binding motif in the modulation of mammalian cell signaling, if any, has not been examined in detail particularly its ability to interact with target membranes or their associated components. In view of this possibility, it is important to label the protein with an environmentally sensitive fluorophore and examine the changes in the fluorescence intensity of the fluorescence reporter group. Such studies have given valuable information regarding penetration of water soluble into the mammalian cell membranes by bacterial toxins [11, 12]. In the present study, the membrane penetration ability of single chain recombinant Jacalin (rJacalin has identical sugar specificity to that of native two-chain jacalin), that has been engineered to contain a lone cysteine residue at the amino acid position 126 within the Caveolin-1 binding motif to understand its interaction with target cell membranes.

The single cysteine containing rJacalin has been labeled with badan, an environmentally sensitive fluorophore whose fluorescence emission intensity increases when

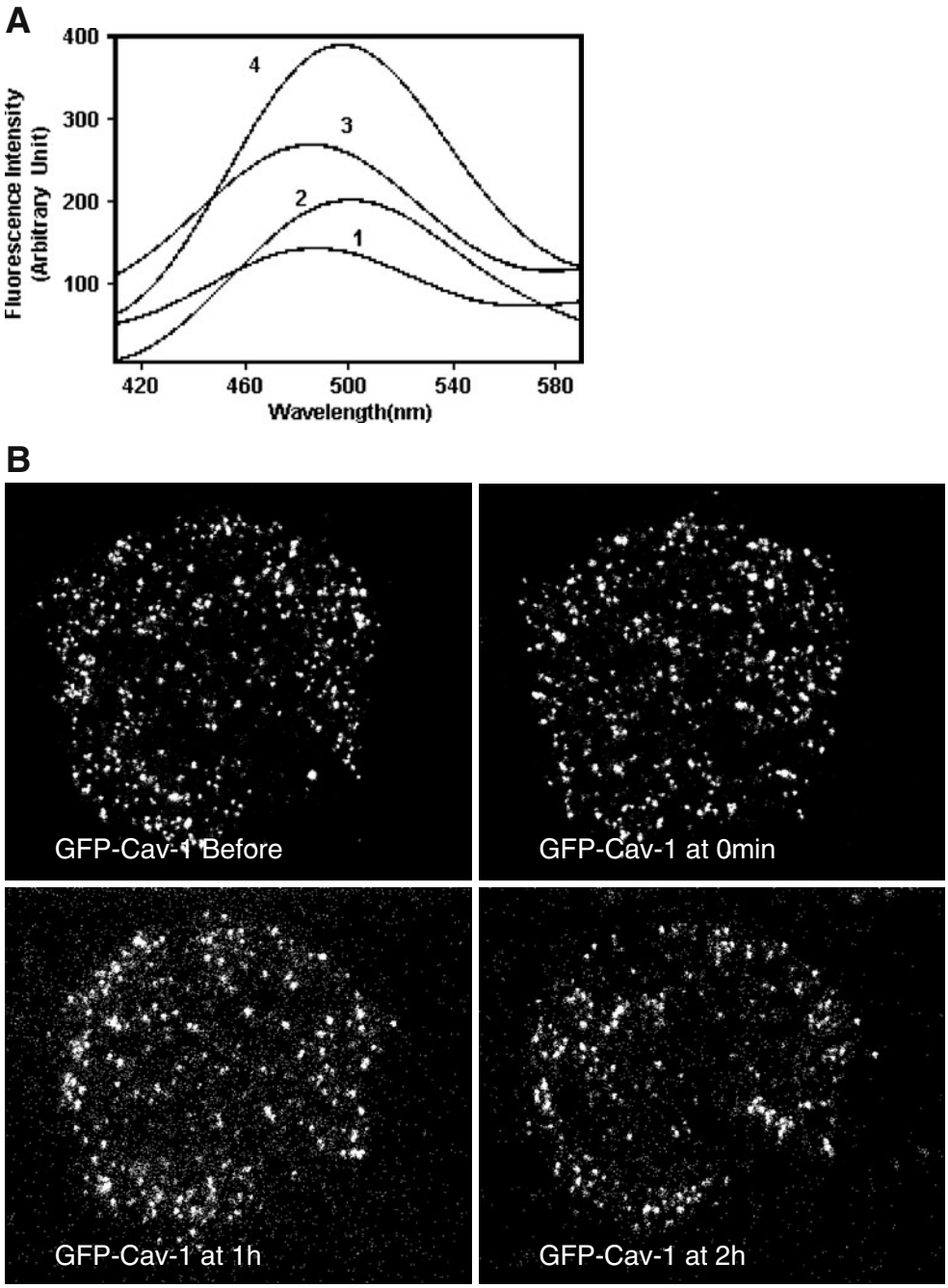

Fig. 1 **A** Membrane penetration of Caveolin-1 biding motif Jacalin: Relative emission spectra of badan labeled rJacalin on rRBCs were measured as described for "Material and methods" section. Various spectra represents all labeled rJacalin at 0 min (1), rJacalin (5 μg/ml) agglutinated total rRBCs after 1 h (3), rJacalin (5 μg/ml) agglutinated pellet in presence of 100 mM Methyl-α-D-Galactoside 1 h (2) and rJacalin (5 μg/ml) agglutinated rRBCs solution in presence of 200 mM Methyl-α-D-Galactoside 1 h(4). **B** TIRF images of Caveolin-1-GFP on HeLa cell basal membrane on Jacalin treatment. Still frame images from the videos showing the distribution of Caveolin-1-GFP before and after treatment of Jacalin (2 μg/ml). Videos were acquired with cascade 512B camera for 200 frames (3 s). Full videos have been provided as "Supplementary material". Data, shown in all panel, is one of the three independent experiments

present in hydrophobic environment in comparison to hydrophilic environment. The fluorescence emission of badan was observed in the presence of erythrocyte membrane to understand the penetration, if any, by the Caveolin-1 binding motif. The data in Fig. 1A clearly shows that the fluorescene of badan has increased by four fold, when badan labeled rJacalin was incubated with rabbit erythrocytes in the presence of 200 mM Methly-alpha-Galactoside. This increase in fluorescence intensity was accompanied by blue shift indicating the specificity of the binding. In comparison, the absence of Methyl-α-Galactoside has resulted in a two fold increase in the fluorescence of badan moiety. This observation suggests that the penetration of the Caveolin-1 binding motif of Jacalin into the target cell membrane in the presence of a competing sugar. In other words Jacalin can predominantly interact through protein-protein or protein-membrane interactions in the presence of competing sugar. Hence, among many possibilities, we are interested in knowing the molecules with which Jacalin interacts on the cell membrane: how this interaction influences the molecules down stream and also the molecules that sense the binding of Jacalin?

It has been reported earlier that during stress, there was an increase in the phosphorylation level of Caveolin-1 and the phosphorylation of caveolin-1 is important for Caveolin-1-mediated signaling events and internalization of caveolae [13]. Microscopic visualization of caveolae suggest that the caveolae domains exits in two states; one in which the caveolae are inactive with respect to membrane trafficking, while the other undergoes fission and fusion events with the membrane as well as involved in long-range transport. It has been reported that in resting cells, 20–30% of the caveolae are dynamics in the local cycling at the plasma membrane, but the portion of activated caveolae could be doubled by addition of SV40, or pharmacologically by inhibition of phosphatase [14, 15]. In view of the above perspective, we employed total internal reflection fluorescence microscopy (TIRFM) to examine the dynamics of the Caveolin-1 upon Jacalin treatment. In order to understand the dynamics of caveolin-1, the HeLa cells were transfected with GFP-Caveolin-1 and studied the dynamics of 'fission and fusion events' or kiss and run ('K&R') dynamics with the plasma membrane. The video presented in SV1, show an even distribution of GFP-Caveolin-1 throughout the cell surface, while the dynamics of GFP-Caveolin-1 seen in the video are consistent with earlier studies [14]. In untreated, GFP-Caveolin-1 transfected HeLa cells, the caveolae were found to undergo 'K&R' with the cell membrane, where it appears and disappears, as seen by their rapid movement (Fig. 1b, video SV1). Interestingly, after 1 h of Jacalin treatment (2 μg/ml), we observed fractional withdrawal of GFP-Caveolin-1 from the cell surface, while the caveolae that were visible showed much constrained movement (confined restricted motion) in contrast to the untreated cells (Video SV2 *vs*. Video SV1, Fig. 1B). As seen from the Video SV3, the fractional loss of fluorescence intensity upon Jacalin treatment may be due to the internalization of GFP-Caveolin-1. After 2 h of Jacalin treatment (2 μg/ml), GFP-Caveolin-1 was found to be arrested mostly at the edges of the cells without any noticeable movement (Video SV3, Fig. 1B). Distribution and dynamics of caveolae have been observed to be affected by the treatment with different molecules. Recently, we had also reported that Taxol, 10-deacetylbaccatinIII, BaccatinIII, Nocodazole and EGF have very different effects on the dynamics of the Caveolin-1 [16]. At this point it becomes very much comprehensible that dynamics of Caveolin-1 act in a different way to an assortment of stimulants and a different downstream signal is conveyed, which further decide the fate of the cell. Probably, it may be the stress signal in case of Jacalin treatment, which causes the movement of the GFP-Caveolin-1 to the cell edges with much restricted movement.

After monitoring the effect of Jacalin on the dynamics of the caveolin-1, lead us to investigate some of the caveolin-1 interacting molecules. It has been reported that PP2A acts as a negative regulator in ERK1/2 signaling upon Jacalin treatment in HT29 cells [6]. It has also been shown that PP2A has a consensus Caveolin-1 binding motif (ΦXXXXΦXXΦ where Φ is Trp or Tyr or Phe) in its catalytic subunits. It was suspected that the Caveolin-1 binds to the subunits of PP2A through the Caveolin-1 scaffolding domain and is presumed to disturb the normal catalytic functions of the enzyme [17]. Recently, it has also been reported that PP2A was targeted to caveolae of cardimyocyes [18]. Incase of HCT116 (colon cancer) cells and over expression of Caveolin-1 was found to increase the amount of phosphorylated Akt by inhibiting the activity of Akt-negative regulators PP1 and PP2A [19]. In view of the association between PP2A and Caveolin-1, we examined the distribution of Caveolin-1 and PP2A. In untreated cells, Caveolin-1 was found to localize uniformly on the cell membrane and uniform cytoplasmic distribution of PP2A but we could not observe any significant co-localization of PP2A with Caveolin-1. However, after 1 h of Jacalin treatment, Caveolin-1 was found to be predominantly at edges of cell-cell contact regions (Fig. 2) also seen by TIRFM on GFP-Caveolin-1 transfected HeLa cells (Fig. 1B) and a remarkable posting of PP2A at the perinuclear space (Figs. 2 and 3C). There were several reports where PP2A has been shown to exert inhibitory as well as stimulatory effects on the growth of cells. Differences in the final outcomes could be due to the differences in the complexes of PP2A with respect to different sub cellular locations and diverse substrate specificities [20, 21]. Consequently, we examined the influence of PP2A on ERK1/2 signaling in A431 cells upon Jacalin treatment.

PP2A, normally associated with PHAP1, was identified as one of the proteins from elute of Jacalin-sepharose column of HT29 cell lysate. In addition, Jacalin treatment has resulted in the phosphorylation of PHAP1 and this phosphorylation was necessary for the release of PP2A from PHAP1 complex [6, 9]. Our study is also in agreement with the dissociation of PP2A/PHAP1 complex after jacalin treatment in A431 cells. Immunoprecipitation with anti-PHAP1 antibodies on untreated A431 cells lysate pulled down the PP2A, evidently exemplify the association of PP2A and PHAP1, while immunoprecipitation with anti-PHAP1 antibodies in Jacalin treated A431 cells lysate does not pull down the PP2A (Fig. 3A). Further, by suppressing the expression level of PP2A using siRNA, we found that the Jacalin treatment has not changed the phosphorylation status of ERK1/2 in comparison to the phosphorylation status of ERK1/2 of unstimulated A431 cells. While, in the absence of siRNA there is a significant decrease in the phosphorylation of ERK1/2 upon Jacalin treatment (Fig. 3B). This clearly highlights the involvement of PP2A in ERK signaling. Moreover, within an hour of

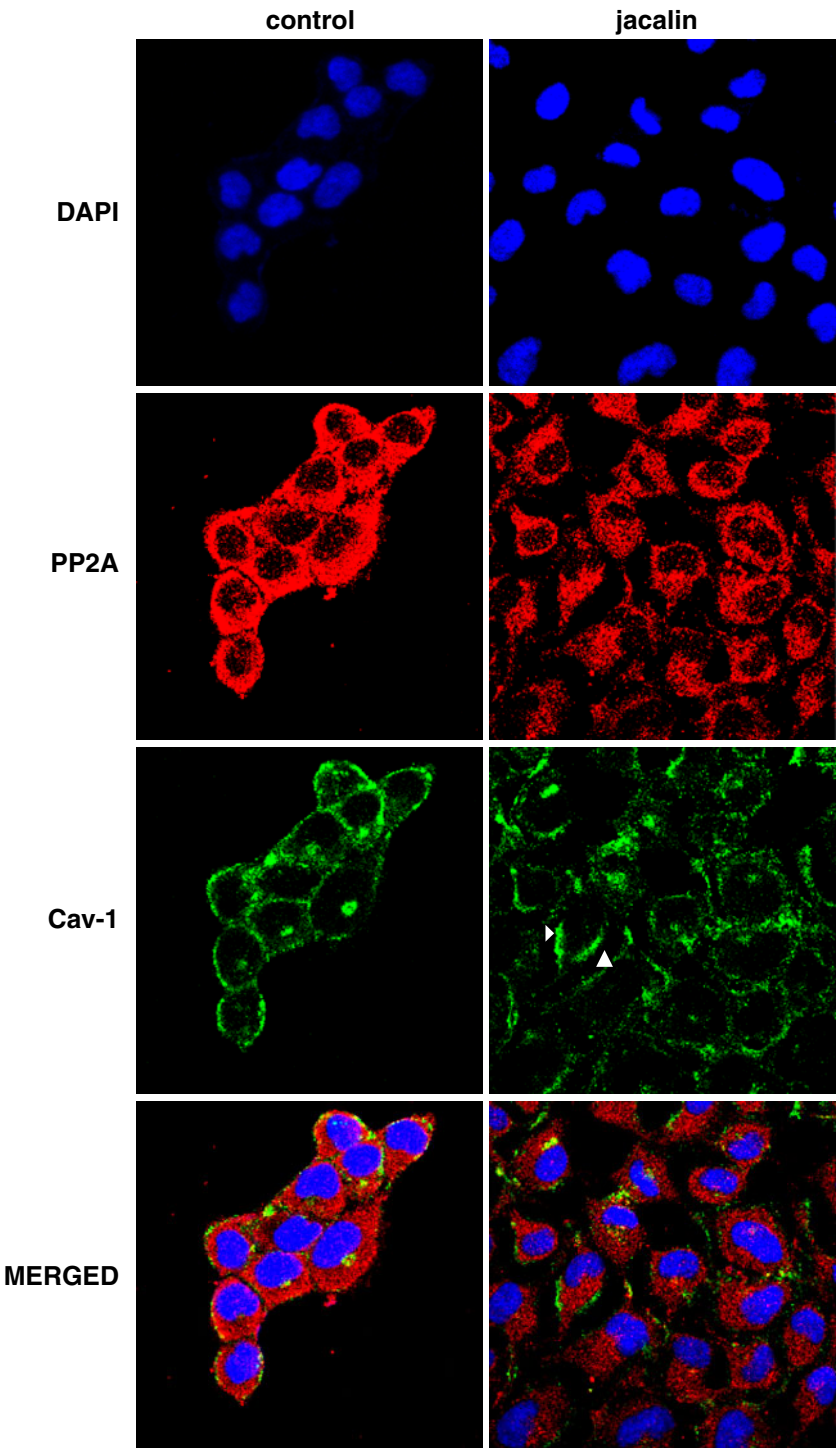

Fig. 2 Effect of Jacalin on cellular localization of PP2A and Caveolin-1. A431 cells were plated on cover slips and processed for confocal imaging after staining with antibodies against PP2A and Caveolin-1 for untreated (U) and treated with Jacalin 40 µg/ml for 1 h (Jac).Data is one of the three independent experiments

Jacalin treatment there was a significant decrease in the phosphorylation of MEK1/2, which is upstream of ERK1/2 (Fig. 4A). Jacalin treatment also results in the decreased phosphorylation of Akt that was reported to be the target substrate of PP2A [22]. Akt activation takes place by phosphorylation of T308 and S473 by PI3 kinases, which directs its relocalization. Phosphorylation at both T308 and S473 were thought to be necessary for full activation of the enzyme. Upon Jacalin treatment there was a significant decrease in the phosphorylation of T308 than S473 of Akt (Fig. 4A). Recently, it has been shown that PHLPP was mainly involved in the dephosphorylation of S473 while PP2A mainly dephosphorylates T308 [23]. Hence, it is more likely that PP2A plays an important role in the dephosphorylation of T308 Akt. PP2A was also shown to regulate the activity of Raf-1 by dephosphorylation of

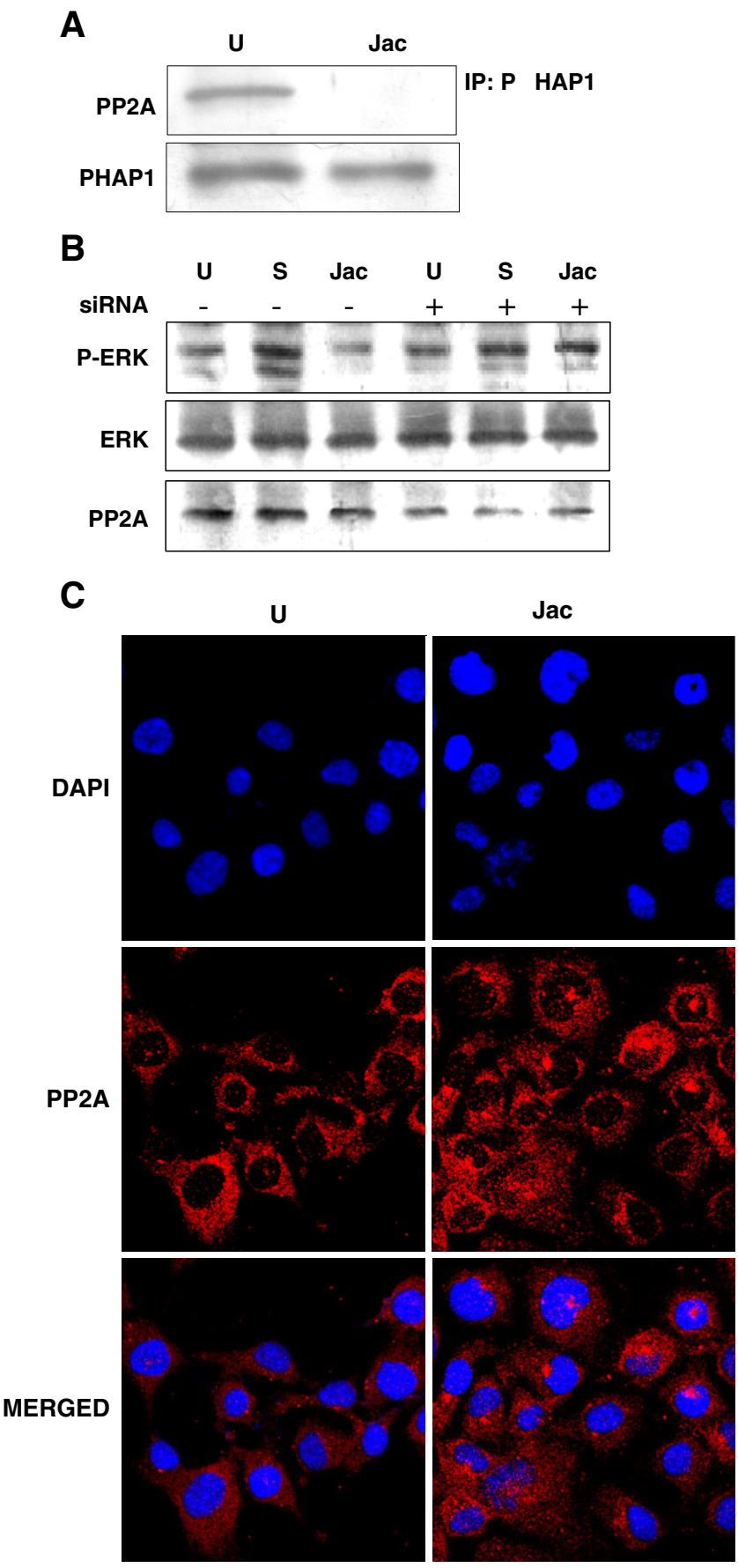

Fig. 3 Activation of PP2A and its redistribution: **A** Association and dissociation of PP2A with PHAP1 in the presence of and absence of Jacalin: Lysates of A431 cells untreated (U) or treated (Jac) with Jacalin were immunoprecipitated with anti-PHAP1 antibodies and probed with anti-PP2A antibodies (upper panel) and anti-PHAP1 antibodies (*lower panel*). **B** Silencing of PP2A expression in A431 cells: The expression of PP2A was silenced with siRNA specific for the same as described in methods section. The cells silenced for PP2A were treated without or with Jacalin and lysates were prepared for examining the phosphorylation levels of p-ERK. The various lanes represent U (Untreated), S (Stimulated with TGFα as positive control) and Jac (treated with Jacalin) in the presence (+) and absence (−) of siRNA against PP2A. All blots were probed with indicated antibodies such as anti-phospho-ERK1/2 (*upper panel*), anti-ERK1 antibodies (middle panel) and anti-PP2A antibodies (*lower panel*). Data is one of the three independent experiments. **C** Perinuclear redistribution of PP2A after Jacalin treatment. A431 cells were plated on cover slips and processed for the confocal imaging after staining with anti-PP2A antibodies on untreated (U) and treated with Jacalin 40 μg/ml for 1 h (Jac)

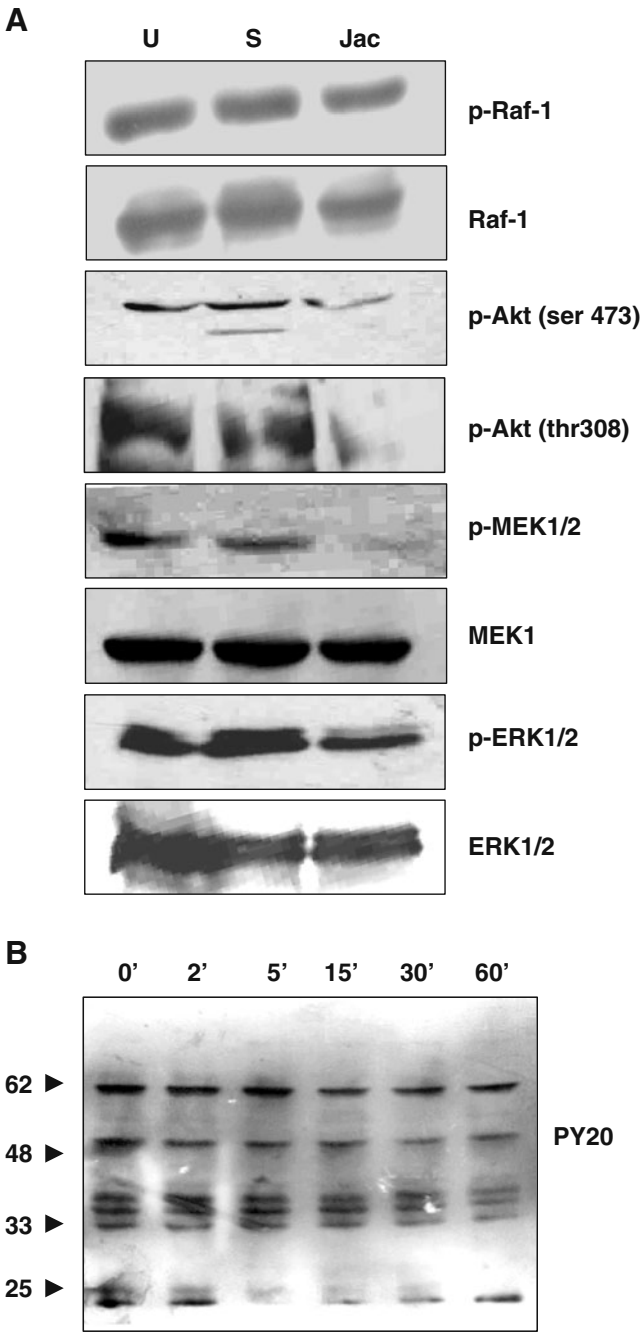

Fig. 4 Effect of Jacalin on MAPK, Akt activities and total cellular tyrosine phosphorylation. **A** Phosphorylation profiles of Raf-1, Akt, MEK1/2 and ERK1/2: A431 cells untreated (U), Stimulated with TGFα as positive control (S) and treated with 40 μg/ml Jacalin (Jac) for 1 h were lysed and 40 μg/lane of proteins were separated by 10% SDS-PAGE followed by immunoblotting against indicated antibodies. **B** Effect of Jacalin on total cellular tyrosine phosphorylation. A431 cells treated with 40 μg/ml Jacalin for indicated times (minutes) were lysed and 40 μg/lane of proteins were separated by 10% SDS-PAGE followed by immunoblotting with antibodies against phosphotryosine antibodies (PY20). The positions of the molecular weight markers are indicated with *arrows* on left hand side blot. Data is one of the three independent experiments

phosphorylated S259 [21, 24]. However, we could not find any change in the phosphorylation of Raf-1 (Fig. 4A). Upon Jacalin treatment, it is not only the PP2A (serine/theronine phosphatase) was found to be active, but also activated protein tyrosine phosphatase(s), as we could find a significant decrease in the overall tyrosine phosphorylation within 1 h treatment (Fig. 4B).

Earlier studies on effects of Jacalin on the proliferation of HT29 revealed that it is a non-cytotoxic inhibitor of colon cancer cells. Jacalin treatment resulted in decrease in the phosphorylation of ERK1/2 in HT29 cells in which PP2A was found to act as a negative regulator in ERK1/2 signaling. However, the linkage between Jacalin and PHAP1 complex is not clear yet. In spite of having different responses of Jacalin on HT29 and A431 cells, it was found that Jacalin treatment results in decrease in the phosphorylation of ERK1/2. In summary, it is important to understand whether the activation of PP2A upon Jacalin treatment is cell type specific or is it a generalized phenomenon applicable to other cells also. In addition, it is still not known about the nature of the proteins that Jacalin interacts on the surface of cancer cells. Hence, an attempt has been made to identify the putative molecule(s) of A431 cells that bind or interact with Jacalin and their involvement in the activation of PP2A, if any.

Proteins of A431 cells that interact or likely to interact with Jacalin were identified from the eluted fractions of Jacalin-sepharose column by mass spectrometry. To our surprise, the proteins eluted from Jacalin-sepharose column were found to be predominantly either ER chaperons like ORP150, Hsp70, Grp78, Glucosidase II or other ER resident proteins. Out of the proteins identified three were found to be phosphorylated at the tyrosine residue (Fig. 5, Table 1). This is the first report in which we have seen an

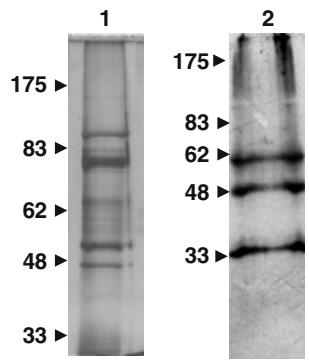

Fig. 5 Protein profile and the phosphorylation status of A431 cell lysate proteins eluted from Jacalin-Sepharose column: **Lane 1** SDS-PAGE gel (8%) pattern of the A431 cell lysate proteins eluted from Jacalin-Sepharose column. **Lane 2** Proteins shown in (**Lane 1**) were probed with anti-phosphotryosine antibodies (PY20). The positions of the molecular weight markers are indicated with arrows on left hand side of each blot. Data shown is one of the three independent experiments

Table 1 Protein identified by mass spectrometry from the elutes of Jacalin-Sepharose column

Name of the protein	No. uniq seqs	Cellular location
150 kDa oxygen-regulated protein ORP150[a]	5	ER
kappa-B motif-binding phosphoprotein—mouse	3	
Alpha-2-HS-glycoprotein precursor (Fetuin-A) (Asialofetuin)	5	secreted
Human LAMP-1	8	PM
nucleobindin 1	2	ER
peroxiredoxin 2	1	ER
peroxiredoxin 1	4	Cyto/Nuc
78 kDa glucose-regulated protein	13	ER
Peroxiredoxin 3, isoform a precursor	1	ER
BiP, heat shock protein 3	2	ER
Chain A, Lumenal Domain Of Calnexin	1	ER
Calnexin	1	ER
108 K heat shock protein	1	ER
Hnrnp A1 (Rbd1,2)	2	
ATPase beta	1	ER
Glucosidase II	37	
hTGN46	1	Golgi
sarco/endoplasmic reticulum Ca2+ -ATPase isoform e	1	ER
MTHSP75	12	ER
TXNDC5 protein	17	ER
KIAA0573 protein	10	ER
Human Ornithine Aminotransferase Complexed With L-Canaline	1	Cyto/Mito
ACAT1 protein	2	Mito
hornerin precursor	1	
Heat-Shock Cognate 70 Kd Protein (44 Kd Atpase N-Terminal Fragment)	7	ER
protein kinase C substrate 80 K-H isoform 2	12	ER
similar to protein phosphatase 1, regulatory (inhibitor) subunit 1A; protein phosphatase inhibitor-1	3	Cyto
Annexin A2:	1	Cyto
Chain ACathepsin D	2	Cyto
78 kDa gastrin-binding protein	4	PM
similar to heterogeneous nuclear ribonucleoprotein A2/B1 isoform 2 [Mus musculus]	3	
desmoplakin [Mus musculus]	1	Desmo
GTP-binding protein	1	
Plakoglobin	2	Desmo
Plakoglobin	1	Desmo

All sequences were found to be proteins of human origin except where mentioned

ER Endoplasmic reticulum; *Cyto* Cytoplasm; *Mito* Mitochondrial matrix; *Nuc* Nucleus; *Desmo* Desmosomes

[a] Reported in Anagh *et al*. (2006)

extensive binding of ER proteins to a plant lectin. A single report describing the binding of N-terminal truncated form of ORP150 to the anti-proliferative *Agaricus bisporus* lectin (ABL). This cytoplasmic form of ORP150 has the carbohydrate ligand, Sialyl-alpha-(2->3)Gal-beta(1->3) GalNAc-alpha, which was shown to be essential for nuclear localization sequence-dependent nuclear protein import. ABL was found to block the nuclear import by binding to the carbohydrate ligand of ORP150 [25]. We also found the presence of ORP150 protein in the fractions of Jacalin-sepharose column from the lysates prepared with A431 cells. But unlike the ABL, which was found to internalize in HT29 cells after binding, Jacalin localizes only to the cell surface in case of A431 cells [4]. There is a possibility that Jacalin affects the A431 cell proliferation by interacting directly or indirectly with some of the chaperones (shown in Table 1) present on the cell surface. However, at this stage the role of cell surface chaperones

in cell proliferation is beginning to be understood. There are several reports highlighting the presence of chaperones Grp78, Grp75, Hsp70, Hsp60, Hsp54 and ORP150 on the surface of several cancerous cells [26]. In line with this observation, we also detected the presence of ORP150 on the cell surface of A431 cells. In this immunofluorescence study ORP150 antibodies was incubated to A431 cells without permeabilization and after permeabilization with NP-40. We can clearly see the membrane localization of ORP150 in untreated and non-permeabilized A431 cells, but in case of Jacalin treatment and non-permeabilized, the intensity of ORP150 is low as compared to Jacalin

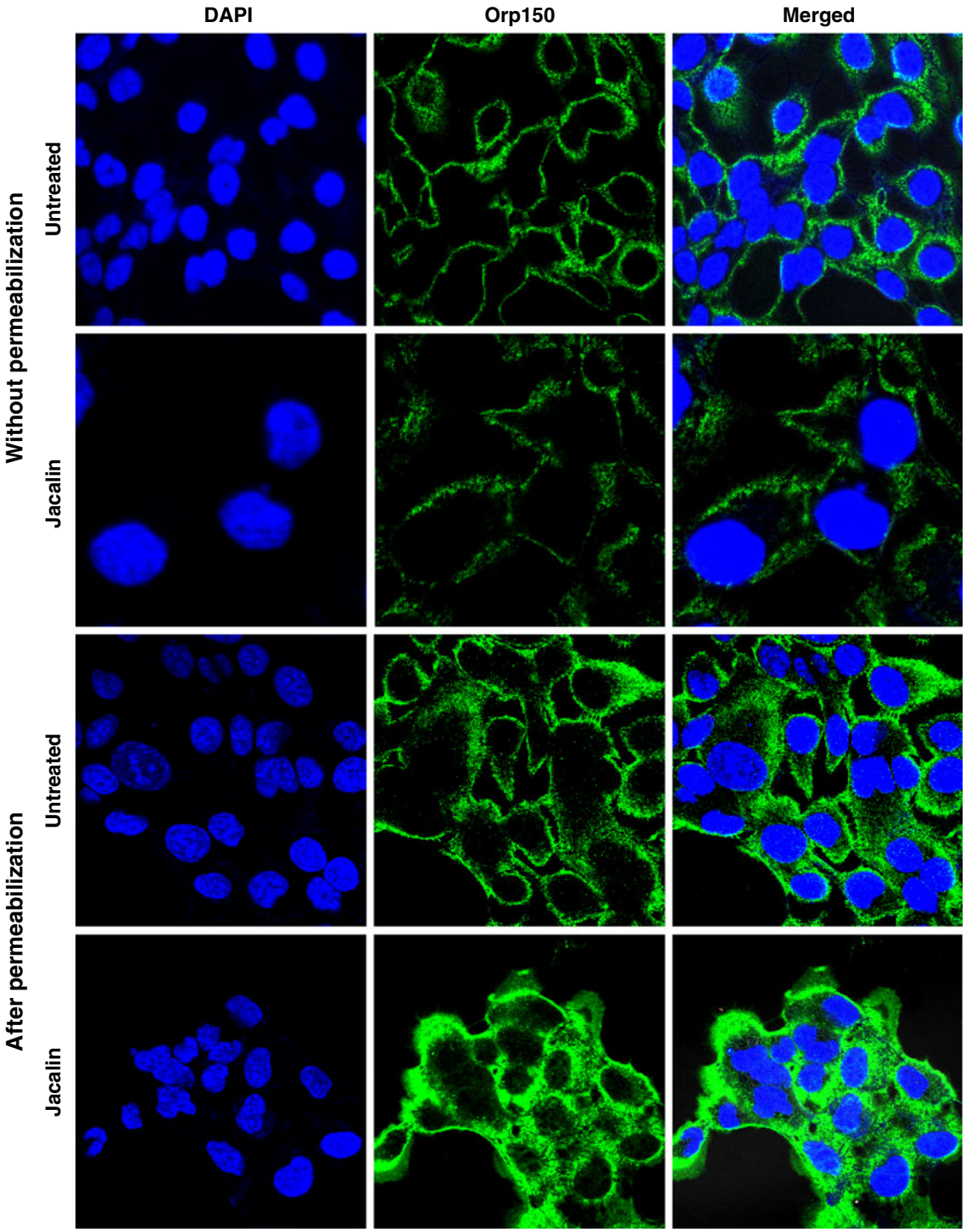

Fig. 6 Confocal microscopic visualization of Orp150: A431 cells were plated on coverslips and processed for confocal imaging after staining with anti-ORP150 antibodies with and without permeabilization. Data shown is one of the three independent experiments

untreated and non-permeabilized A431 cells. It may be due to the withdrawal of the ORP150 from membrane to the cytoplasm upon Jacalin treatment. Withdrawal of ORP150 to the cytoplasm upon Jacalin treatment was further confirmed after seeing the distribution of ORP150 with permeabilization. Upon staining untreated A431 cells with ORP150 antibodies show both membrane and cytoplasmic distribution, however, after Jacalin treatment for 1 h there is enhanced cytoplasmic localization of ORP150 (Fig. 6). At this point we can certainly say the Jacalin treatment has resulted in the withdrawal of ORP150 from membrane to cytoplasm. In our earlier work also we have reported that overexpression of ORP150 in A431 cells has resulted in a dramatic protection of A431 cells against Jacalin-induced toxicity, confirming that the Jacalin-induced cytotoxicity is mediated through ORP150, and impairment of ORP150 functions with the help of Jacalin makes the cells more susceptible to death due to stress. There is a recent report that suggests that up-regulation of ORP150 decreases the anti-tumor activity of the drug by inhibiting apoptosis [27]. In continuation of the work here we further confirmed the membrane localization of ORP150. Since, Jacalin has been shown to interact with most of the ER resident proteins including ORP150, Hsp70, and Grp78; therefore, we wished to see if there was any co-localization of PP2A with ER resident proteins. However, no such co-localization could be detected (data not shown). At this stage it is yet not clear whether the changes in the phosphorylation patterns of important cellular proteins by Jacalin are mediated through these stress proteins. Considering the fact that most of the proteins that Jacalin was found to interact and caveolae are originated from the endoplasmic reticulum, it will be interesting to understand the link between ER stress chaperones and caveolae vesicles that are modulated by Jacalin treatment.

In summary, Jacalin induced cytotoxicity is observed with dramatic change in the dynamics of the Caveolin-1 and its distribution along with the activation of PP2A and its redistribution. Our studies also illustrate ORP150 as one of the molecules that interacts with the Jacalin and membrane localization of ORP150 along with cytoplasmic distribution. Further it will be interesting to identify the natural ligands of ORP150 that it assists on the membrane under *in vivo* condition and its linkage with the endycytotic vescicles and/or caveolae in general.

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