Accumulate Phosphatidylinositol 4,5-Bisphosphate*

Syndrome Lack OCRL Inositol Polyphosphate 5-Phosphatase and Accumulate Phosphatidylinositol 4,5-Bisphosphate

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5-phosphatase isoforms or differences in tissue distribution or subcellular localization account for the lack of compensation.

We have now compared the activities of full-length and truncated OCRL encoding the region that is homologous to 5-phosphatase II. We find that they have similar activities using all four substrates, suggesting that the enzyme activity of OCRL protein is determined by its 5-phosphatase II homology region. We have used kidney proximal tubule cell lines from a patient with Lowe syndrome compared with control cell lines from a normal individual to study the expression of OCRL and other 5-phosphatases, phosphatidylinositol metabolism, and the subcellular localization of OCRL. We find that OCRL is absent in Lowe syndrome cells and is the major PtdIns 4,5-P2 hydrolyzing enzyme in normal kidney proximal tubule cell lines and that there is accumulation of PtdIns 4,5-P2 in the Lowe syndrome cells. We also find that OCRL is associated with lysosomes in control cells.

EXPERIMENTAL PROCEDURES

Materials—[3H]Inositol, [3H]Ins1,3,4,5-P4, [3H]PtdIns 4,5-P2, and γ-[32P]ATP were purchased from NEN Life Science Products. PtdIns was purchased from Avanti. PtdIns 4,5-P2 standard was from Boehringer Mannheim. [3H]Orthophosphate was from ICN, and 32P-labeled Ins 1,4,5-P2 was prepared as described (12). The plasmid ps.OCRL-B-1 was kindly provided by Robert Nussbaum (National Institutes of Health). The pVL1393 baculoviral expression vector and BacculoGold transfection kit were from PharMingen. Anti-SHIP antibody was a gift from Gerald Krystal (The Terry Fox Laboratory, Vancouver, British Columbia, Canada). Anti-5-phosphatase I, anti-5-phosphatase II, and anti-OCRL antibodies were the same as described (8, 13, 14). Monoclonal anti-protein disulfate isomerase was purchased from Affinity Bioreagents. Monoclonal anti-protein disulfate isomerase was purchased from Affinity Bioreagents.

Bacterial cloning was carried out in the Escherichia coli strain DH5α. Insertion in a EcoRI site in the P1 expression vector (p1E3) was accomplished by the inverse PCR method described previously (18). The GroPtdIns products of deacylation were mixed with [3H]Ins 1,4,5-P3, 32P-labeled Ins 1,4,5-P3, and various amounts of enzyme. The reactions were performed at 37 °C for times ranging from 0 to 60 min. The reactions were stopped by addition of 80 μl of chloroform:methanol (1:1), and the lipid products were extracted by vortexing and separated as described (16). The products of reactions were detected by autoradiography. The spots were cut out and counted using a Beckman scintillation counter.

Molecular cloning of cDNA inserts of ps.OCRL3 and ps.OCRL4 were subsequently subcloned into baculoviruses encoding full-length OCRL and a truncated OCRL lacking Met264 of OCRL1 as described previously (8). The ps.OCRL4 contains OCRL cDNA starting from the translation initiation region to exclude mutations (Sequenase 2.0, United States Biochemical Corp.). The ps.OCRLB-1 was kindly provided by Robert Nussbaum (National Institutes of Health).

Subcellular Localization of OCRL—We find that OCRL is absent in Lowe syndrome cells and is the major PtdIns 4,5-P2 hydrolyzing enzyme in normal kidney proximal tubule cell lines and that there is accumulation of PtdIns 4,5-P2 in the Lowe syndrome cells. We also find that OCRL is associated with lysosomes in control cells.

Fractionation of 5-Phosphatase Isozymes in NHK and LS Cells and 5-Phosphatase Assays—The same numbers of NHK and LS cells were each seeded in five p100 Primaria dishes and allowed to grow to 90% confluence. The cells were harvested in PBS, resuspended, and extracted as described above. The protein concentration of the extracts was measured using Bio-Rad protein assay reagent (Bio-Rad). The same amount of total protein (3 mg) from NHK and LS cell extracts was diluted to 10 ml immediately before loading onto a 1-ml Mono S column equilibrated with buffer A (25 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM MgCl2, and 0.05% Triton X-100) for purification of OCRL protein. The column was eluted at 1 ml/min with buffer B (buffer A plus 0.5 M NaCl) programmed as follows: 0–2 min, buffer A; 2–32 min, 0–100% buffer B. The peak OCRL fractions were identified by assaying for Ins 1,4,5-P3 5-phosphatase activity and by Western blotting using anti-OCRL antibodies. The equivalent fractions obtained from extracts of Lowe cells infected with MEG-01 were used as blanks in 5-phosphatase assays.

Phosphatidylinositol bisphosphate accumulates in the lysosomal fraction of human kidney, liver, and spleen, and is the major PtdIns 4,5-P2 hydrolyzing enzyme in normal kidney proximal tubule cell lines and is the major PtdIns 4,5-P2 hydrolyzing enzyme in normal kidney proximal tubule cell lines. We find that OCRL is absent in Lowe syndrome cells and is the major PtdIns 4,5-P2 hydrolyzing enzyme in normal kidney proximal tubule cell lines and that there is accumulation of PtdIns 4,5-P2 in the Lowe syndrome cells.
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RESULTS

The 5-Phosphatase II Homology Domain of OCRL Protein Determines Its Enzyme Activity—We have previously characterized a 90-kDa amino-terminal truncated OCRL protein that encodes the entire domain homologous to 5-phosphatase II (8). We showed that the 90-kDa OCRL is a 5-phosphatase with a substrate preference for PtdIns 4,5-P$_2$. We now compare full-length OCRL to the truncated OCRL protein. We expressed full-length OCRL, the 90-kDa truncated OCRL, and a protein tyrosine phosphatase MEG-01 as a negative control (7, 8) in Sf9 cells followed by a one-step purification procedure using a Mono S column. Peak fractions containing OCRL and the corresponding fraction from MEG-01 extracts were analyzed by SDS-PAGE (Fig. 1A). OCRL was purified to near homogeneity in one step due to the high level of OCRL in Sf9 cells (Fig. 1A). Full-length OCRL migrated as a 105-kDa protein as shown in Fig. 1A, lane 1, whereas the truncated protein migrated at 90 kDa. We compared full-length OCRL to truncated OCRL using all four 5-phosphatase substrates (Fig. 1, B–D). Full-length OCRL hydrolyzed Ins 1,4,5-P$_3$ with a $K_m$ of 123 ± 13 μM and Ins 1,3,4,5-P$_4$ with a $K_m$ of 28 ± 9 μM (data not shown). Truncated OCRL hydrolyzed Ins 1,4,5-P$_3$ with a $K_m$ of 145 ± 31 μM and Ins 1,3,4,5-P$_4$ with a $K_m$ of 25 ± 8 μM (data not shown). These values are similar to those reported previously (8). The activities of purified full-length and truncated OCRL proteins using Ins 1,4,5-P$_3$, Ins 1,3,4,5-P$_4$, and PtdIns 4,5-P$_2$ were less than those reported previously using the 90-kDa OCRL in crude Sf9 cell extracts (8). This parallel reduction of enzyme activity using all the substrates could result from the loss of activity after purification. We find that full-length OCRL has 5-phosphatase activity that is similar to and has the same substrate preference as truncated OCRL. It appears that the 5-phosphatase II homology region of OCRL protein determines its enzyme activity, whereas the amino-terminal region of OCRL is not required for its enzyme activity.

OCRL Is the Major PtdIns 4,5-P$_2$ 5-Phosphatase in Human Kidney Proximal Tubule Cells—A Western blot of the total cell OCRL proteins. $B$, hydrolysis of Ins 1,4,5-P$_3$ and Ins 1,3,4,5-P$_4$ by OCRL proteins. Full-length OCRL (22.5 ng) or truncated OCRL (15 ng) was assayed in a 25-μl reaction mixture containing [3H]PtdIns 1,4,5-P$_3$ (0–150 μM) or [3H]PtdIns 1,3,4,5-P$_4$ (0–50 μM) for 20 min at 37 °C as described (8). $C$, hydrolysis of PtdIns 4,5-P$_2$ by OCRL proteins. Full-length OCRL (22.5 ng) or truncated OCRL (15 ng) was assayed in a 25-μl reaction mixture containing 50 μM [3H]PtdIns 4,5-P$_2$ for the time indicated at 37 °C as described (8). $D$, hydrolysis of PtdIns 3,4,5-P$_3$ by OCRL proteins. Same amount of full-length or truncated OCRL protein as above was assayed at 37 °C for the time indicated as described under “Experimental Procedures.” The background activities in these assays were determined by assaying the same amount of corresponding MEG-01 fractions. Error bars indicate S.D. from at least four experiments.
extract from two NHK cell lines and three LS cell lines using a rabbit anti-OCRL carboxyl-terminal peptide antiserum is shown in Fig. 2. OCRL protein was absent in the LS cell extracts, whereas native OCRL protein was detected in NHK cells and appears to be the same size as recombinant full-length OCRL (Fig. 2). The same Western blot was also probed with a rabbit anti-OCRL amino-terminal peptide antiserum and a rabbit anti-full-length OCRL antiserum, but neither detected immunoreactive OCRL protein in extracts from LS cells (data not shown). The absence of OCRL protein indicates that this Lowe syndrome patient has a loss of function mutation like other Lowe syndrome patients reported (3, 10, 11).

We tested whether other 5-phosphatases are present in these cells. Western blots of the total cell extracts from two NHK cell lines and two LS cell lines using different 5-phosphatase antibodies are shown in Fig. 3. 5-Phosphatase I, 5-phosphatase II, and SHIP 5-phosphatase representing groups I, II, and III 5-phosphatases, respectively, were all present in NHK and LS cells. OCRL is only present in the NHK cells (Fig. 3). The existence of multiple 5-phosphatases in these cells indicates that the renal defect in Lowe syndrome is not due to an absence of 5-phosphatase enzyme per se and suggests that OCRL has a specific function that is not provided by the other 5-phosphatases.

We next investigated the substrate specificity of native OCRL protein in these cells. We fractionated equal amounts of extract from NHK and LS cells using a Mono S column to separate OCRL from other 5-phosphatases as described under "Experimental Procedures." We analyzed 0.3% (v/v) of the flow-through and 3% (v/v) of column fractions by Western blotting with different 5-phosphatase antibodies. Fractions 1–7 and 18–25 contained no detectable OCRL protein (data not shown). Fractions containing immunoreactive OCRL and other 5-phosphatases (fractions 7–18) are shown in Fig. 4. SHIP 5-phosphatase...
tase was found in the flow-through and fractions 10–13 in both the NHK and LS cells (Fig. 4A). The 5-phosphatase I was also found in the flow-through and in fractions 7–13 in the LS cells and fractions 7–18 in the NHK cells (Fig. 4C). The 5-phosphatase II did not bind to Mono S and was found in the flow-through of both NHK and LS cells (data not shown). OCRL in NHK cells was not detected in the flow-through. The peak immunoreactivity was found in fractions 7, 8, 15, and 16 (Fig. 4B). That two peaks of OCRL were eluted from the column suggests that two forms of OCRL are present in these cells. We also observed a truncated form of OCRL in NHK cells in fractions 7–11 (Fig. 4B). This truncated OCRL was cleaved from its amino terminus since it was not detected with an anti-OCRL amino-terminal peptide antibody (data not shown). We did not detect any precursor-product relationship of the truncated OCRL by pulse-chase labeling of NHK cells (data not shown), suggesting that the truncated forms of OCRL were an artifact of in vitro proteolysis.

We then assayed the Mono S column fractions for the ability to hydrolyze all four 5-phosphatase substrates (Fig. 5). The Ins 1,4,5-P3 5-phosphatase activity was found mainly in the flow-through fraction, corresponding to 5-phosphatases I and II that are present in NHK and LS cells (Fig. 5A). The total Ins 1,4,5-P3-hydrolyzing activity in NHK cells is 1.7-fold higher than that in LS cells. This reduced activity in LS cells is possibly due to the lack of OCRL. However, the majority of Ins 1,4,5-P3 hydrolyz-
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TABLE I

| Experiment 1 | Experiment 2 | Experiment 3 | Mean ± S.D. |
|--------------|--------------|--------------|-------------|
| NHK96        | LS5          | NHK96        | LS5         | NHK96        | LS5         | NHK52        | LS8          | NHK96        | LS5         | NHK cells | LS cells |
| PtdIns       | cpm          | % total      | cpm          | % total      | cpm          | % total      | cpm          | % total      | cpm          | % total      | cpm          | % total      |
| PtdIns 3P    | 295,000      | 93.87        | 92.81        | 1,066,714    | 984,303      | 92.04        | 92.49        | 176,353      | 229,038      | 94.44      | 93.17      | 93.5 ± 1.0   | 92.8 ± 0.3   |
| PtdIns 3P    | 760          | 0.24         | 0.19         | 1964         | 2225         | 0.17         | 0.21         | 120          | 272          | 0.06       | 0.11       | 0.2 ± 0.08   | 0.2 ± 0.05   |
| PtdIns 4P    | 13,650       | 4.34         | 4.49         | 58,346       | 43,489       | 5.03         | 4.09         | 8909         | 9541         | 4.33       | 3.88       | 4.6 ± 0.3    | 4.2 ± 0.3    |
| PtdInsP$_2$  | 163          | 388          | 0.05         | 0.10         | 10,598       | 5186         | 0.91         | 0.49         | 180          | 262         | 0.10       | 0.11       | 0.4 ± 0.39   | 0.2 ± 0.18   |
| PtdIns 3,4-P$_2$ | 44  | 42          | 0.01        | 0.01        | 130         | 166         | 0.01        | 0.01        | 24           | 33          | 0.01       | 0.01       | 0.01 ± 0    | 0 ± 0        |
| PtdIns 4,5-P$_3$ | 4631 | 9388       | 1.47        | 2.40        | 21131       | 28,701      | 1.82        | 2.70        | 1973         | 6682        | 1.05       | 2.72       | 1.4 ± 0.3    | 2.6 ± 0.1    |
| PtdIns 3,4,5-P$_3$ | ND$^a$ | ND          |             |             | 111         | 108         | 0.01        | 0.01        | ND           | ND          | 0.01       | 0.01       | 0.01         | 0.01        |

$^a$ ND, none detected.

The majority of Ins 1,3,4,5-P$_4$ 5-phosphatase activity was present in the flow-through of NHK and LS cells (Fig. 5B), suggesting that 5-phosphatases present in the flow-through including 5-phosphatase I, 5-phosphatase II, and SHIP are the major contributors to this activity. The total Ins 1,3,4,5-P$_4$ 5-phosphatase activity was similar in NHK cells and LS cells, suggesting that OCRL does not account for this activity in these cells.

In contrast, PtdIns 4,5-P$_2$ 5-phosphatase activity was barely detectable from LS cells in comparison to NHK cells (Fig. 5C). The total PtdIns 4,5-P$_2$-hydrolyzing activity in NHK cells is 10-fold higher than that in LS cells. The PtdIns 4,5-P$_2$ 5-phosphatase activity in NHK cells correlated with the peak fractions of full-length OCRL (fractions 7 and 8) and the fractions containing truncated OCRL (fractions 7–12) (Fig. 5C). The 5-phosphatase II is another PtdIns 4,5-P$_2$-hydrolyzing enzyme. We were unable to detect its PtdIns 4,5-P$_2$-hydrolyzing activity in the flow-through because its concentration is too low. That amino-terminal proteolyzed OCRL is active is expected since the 90-kDa amino-terminal truncated recombinant OCRL has the same enzyme activity as full-length OCRL. This analysis indicates that OCRL is the major P1 4,5-P$_2$ 5-phosphatase in these cells.

PtdIns 3,4,5-P$_3$ 5-phosphatase activity of these fractions is shown in Fig. 5D. The activity in the flow-through of NHK and LS cells may be due to SHIP and type II 5-phosphatase (Figs. 5B and 5D). The activity in fractions 7, 8, 10, 11, 15, and 16 of NHK cells correlated with the peak fractions of full-length OCRL (fractions 7 and 8) and the fractions containing truncated OCRL (fractions 7–12) (Fig. 5C). As a result, the total PtdIns 3,4,5-P$_3$-hydrolyzing activity in NHK cells is 2.5-fold higher than that in LS cells. Interestingly, there is a peak of activity in fractions 7 and 8 of LS cells that is not due to OCRL (Fig. 5D). Fractions 7 and 8 had little 5-phosphatase activity using other 5-phosphatase substrates (Fig. 5, A–C). Additionally, this activity does not require the presence of MgCl$_2$ (data not shown), suggesting that it is a group IV phosphatase (i.e. the group IV enzymes only hydrolyze PtdIns 3,4,5-P$_3$) (22). OCRL is not the only contributor of PtdIns 3,4,5-P$_3$ 5-phosphatase activity in these cells, although the absence of OCRL reduced overall PtdIns 3,4,5-P$_3$ 5-phosphatase activity in OCRL cells. It is not clear from these experiments whether the two forms of native OCRL have different enzyme activities.

PtdIns 4,5-P$_2$ Metabolism Is Abnormal in LS Cells—The substrate specificity of OCRL suggests that one consequence of loss of OCRL might be abnormal PtdIns 4,5-P$_2$ metabolism. To test whether OCRL cells have an abnormal level of PtdIns 4,5-P$_2$, we performed three $[^3H]$inositol labeling experiments using two NHK cell lines and two LS cell lines. HPLC analysis of glycerophosphorylinositols derived from the inositol lipids is summarized in Table I. HPLC fractionation of glycerophosphorylinositols from two experiments is shown in Fig. 6. PtdIns 4,5-P$_2$ accumulated in LS cells, whereas the levels of other glycerophosphorylinositols were similar in NHK and LS cells.
We could not detect PtdIns 3,4-P₂ and PtdIns 3,4,5-P₃ even when these cells were stimulated with platelet-derived growth factor on the scale that these experiments were carried out. Whether OCRL plays a role in PtdIns 3,4,5-P₃ metabolism remains to be determined. We also observed an unknown glycerophosphorylinositol (GroPtdInsPₓ) molecule that eluted about 7 min earlier than GroPtdIns [³²P]3,4-P₂ standard (Table I). This GroPtdInsPₓ when treated with OCRL did not elute with GroPtdIns [³²P]3-P standard but rather just after GroPtdIns, indicating that it is not a putative PtdIns 3,5-P₂ that has been speculated to exist (19). Our result shows that one renal defect in Lowe syndrome is in PI 4,5-P₂.
metabolism. That 10-fold lower PtdIns 4,5-P$_2$-hydrolysis activity in LS cells results in only 2–3-fold accumulation of PtdIns 4,5-P$_2$ may result from the fact that only part of the PtdIns 4,5-P$_2$ in these cells is regulated by OCRL.

**OCRL Is Associated with Lysosomes in NHK Cells**—We studied the intracellular localization of OCRL. We first fractionated cell extracts into three major fractions: cytosolic, detergent-soluble, and detergent-insoluble fractions. More than 80% of OCRL was associated with the detergent-soluble membrane fractions (data not shown). We did immunohistochemistry using NHK and LS cells and affinity purified anti-OCRL carboxyl-terminal antibody. This antibody stained NHK cells in a peri-nuclear pattern suggesting that OCRL is associated with intracellular membranes, whereas there is no staining of LS cells (Fig. 7, A and B). We then did double-immunostaining using NHK cells with anti-OCRL antibody and antibodies to marker proteins for Golgi (γ-adaptin), endoplasmic reticulum (protein disulfate isomerase), and lysosomes (Lamp1). OCRL co-localized with the lysosomal marker Lamp1 (Fig. 7, C and D), whereas the pattern with OCRL antibody was different from those with either anti-protein disulfate isomerase or anti-γ-adaptin antibodies. Our result is different from the earlier study suggesting that OCRL is localized in the Golgi apparatus (9, 23). To confirm our result, we treated these cells with sucrose to generate sucrosomes (21). Sucrosomes arise from lysosomes because lysosomes of mammalian cells do not contain invertase and thus lysosomes accumulate sucrose (21, 24, 25). The swollen morphology of sucrose-retaining lysosomes is distinctive and allowed us to distinguish lysosomes from other intracellular organelles. Sucrose-treated normal cells were double-stained with anti-OCRL antibody and anti-Lamp1 antibody in comparison to anti-OCRL antibody and anti-γ-adaptin antibody (Fig. 7, E–H). The swollen lysosomes in NHK cells were co-stained by anti-OCRL antibody and anti-Lamp1 antibody (Fig. 7, E and F), whereas anti-γ-adaptin antibody stained a different region that is distinct from the swollen lysosomes (Fig. 7, G and H). We also compared anti-Lamp1 staining of NHK and LS cells before and after sucrose treatment and did not detect any morphological differences. Our result suggests that OCRL may play a role in lysosomal function by regulating PI 4,5-P$_2$ levels in that organelle.

**DISCUSSION**

We have studied cell lines from kidney proximal tubules in an attempt to determine the function of OCRL. We found that OCRL is the major PtdIns 4,5-P$_2$-5-phosphatase in these cells. We found that there was 10-fold less PtdIns 4,5-P$_2$-5-phosphatase activity in kidney tubule cell lines from the Lowe syndrome patient. A similar decrease was also found in fibroblasts from Lowe syndrome patients (9, 11). LS cells lacking OCRL accumulate abnormal levels of PtdIns 4,5-P$_2$, even though at least four other 5-phosphatases are present in these cells. However, only one of the other 5-phosphatases hydrolyzes PtdIns 4,5-P$_2$ (5-phosphatase II), and it apparently does not provide enough activity in the correct cellular locale to correct the defect in these cells. In contrast, 5-phosphatase II enzyme accounts for essentially all PtdIns 4,5-P$_2$ hydrolysis in platelets where OCRL is not present (26), suggesting that there are differences in the metabolism of PtdIns 4,5-P$_2$ in different cell types.

The intracellular localization of a variety of 5-phosphatases has been studied in mammalian cells. 5-Phosphatase I has been found in the cytosol and associates with cell membranes including plasma membrane, endoplasmic reticulum, and Golgi apparatus via carboxyl-terminal prenylation (27). 5-Phosphatase II is also in the cytosol and associates with mitochondria and is also prenylated (28). SHIP has been shown to associate with tyrosine kinase receptors (29–31) and therefore most likely cycles between cytosol and plasma membrane. We examined the intracellular localization of OCRL in the kidney proximal tubule cell lines. We observed that OCRL is associated with lysosomes in these cells, which is distinct from the localization of other 5-phosphatases. Therefore, the subcellular localization of OCRL may represent another factor that contributes to the inability of other 5-phosphatases to compensate for the defect in Lowe syndrome. Our intracellular localization of OCRL is different from the Golgi apparatus localization of OCRL reported earlier (9, 23). This discrepancy may be due to the use of different cell types for localization: fibroblasts were used previously, whereas the current study utilizes kidney cells. We have measured the activity and secretion of lysosomal enzymes in these kidney proximal tubule cell lines. LS cells had a significantly decreased level of lysosomal enzymes including cathepsin B, cathepsins B + L and β-hexosaminidase relative to NHK cells. Consistent with this result, LS cells secreted an abnormally high proportion of lysosomal enzymes extracellularly. These results suggest that OCRL plays a specific role in regulating the trafficking of lysosomal enzymes.

Lysosomes have been considered as a terminal degradative compartment of cells. However, recent studies have shown that lysosomal membranes recycle actively using vesicular intermediates (21, 32–35). The most direct evidence for this comes from the observation that normal polyhedral clathrin coats nucleated by AP-2 adaptor can form on mature lysosomes under physiological conditions in vitro (21), suggesting that clathrin-coated vesicles might mediate retrograde membrane traffic out of lysosomes. PtdIns 4,5-P$_2$ has been shown to be involved in the regulation of vesicular traffic (for review, see Ref. 36). Specifically, it has been shown to bind AP-2 and dynamin, both of which mediate clathrin coat formation (36–38). It is possible that OCRL functions in lysosomal membrane trafficking by regulating the specific pool of PtdIns 4,5-P$_2$ that is associated with lysosomes, such that a misregulation due to the loss of OCRL function would lead to abnormal delivery of lysosomal enzymes to extracellular compartments. It will be interesting to determine whether the PtdIns 4,5-P$_2$ associated with lysosomal membranes accumulates to an even greater extent in LS cells than that which we detected as an elevation in total cellular PtdIns 4,5-P$_2$.

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