Glutathione Conjugates Recognize the Rossmann Fold of Glyceraldehyde-3-phosphate Dehydrogenase*

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Leukotriene (LT) C4 and other glutathione conjugates are synthesized intracellularly and then move to the plasma membrane for export. The intracellular proteins that bind these molecules and the significance of these interactions are poorly understood. To identify the binding sites of membrane-associated proteins that recognize these molecules, we utilized photoaffinity probes to label the inner leaflet of erythrocytes. The predominant molecule labeled with S-(p-nitrobenzyl)glutathione-[125I]4-azidosalicylic acid (PNBG-[125I]ASA) or LTC4-[125I]4-azidosalicylic acid (LTC4-[125I]ASA) was 38 kDa. The protein was labeled with PNBG-[125I]ASA, electrolabeled to polyvinylidene difluoride membranes, digested in situ with lysyl endopeptidase, and two radiolabeled peptides isolated by reverse phase-high performance liquid chromatography. These contained an identity of 7/11 with amino acids 119–129, and 11/11 with amino acids 87–77 of human liver glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively. Photoaffinity labeling with PNBG-[125I]ASA was blocked completely by 100 \( \mu \)M NAD\(^+\) and greater than 50% with 100 \( \mu \)M NAD\(^-\). LTC4-[125I]ASA binding to the NAD\(^-\) site was confirmed by V8 protease digestion of purified GAPDH labeled with LTC4-[125I]ASA or PNBG-[125I]ASA, with both labels localized to the 6.8 kDa N-terminal fragment. Photoaffinity labeling of HL-60 cells with LTC4-[125I]-ASA identified GAPDH as the predominant cytoplasmic binding protein in these cells. These data indicate that GAPDH is a membrane-associated and cytoplasmic protein which binds glutathione conjugates including LTC4.

Leukotriene (LT) C4 and the biologically active prostaglandins (PG) are synthesized at specific intracellular locations and then move to sites where they are exported from cells. 5-Lipoxygenase is targeted to the nuclear membrane (1–3) where, in conjunction with 5-lipoxygenase-activating protein (FLAP), it catalyzes the sequential conversion of arachidonic acid to both (5S)-HETE and to LTA4 (4, 5). LTA4 is then conjugated with either reduced glutathione to form LTC4 by the enzyme LTC4 synthase (6–8), or acted on by the enzyme LTA4 hydrolase to form LTD4 (9). Based on the structural similarity between LTC4 synthase and FLAP, the intracellular location where this reaction most likely takes place is on the inner leaflet of the nuclear membrane (8). LTC4 formed at this location must then traverse the cell until it comes into association with the multidrug resistance-associated protein (10, 11), located in the plasma membrane (12), which mediates its export and that of other glutathione conjugates.

The requirement for intracellular movement and subsequent export is shared with arachidonic acid metabolites of the PG family. PGH synthase-2 is located with its active site facing the interior of the nucleus (13, 14), so that PGH2 formed at this site must be exported from the nucleus before it is acted on by enzymes catalyzing the formation of the bioactive PGs (e.g. PGE2 and PGD2). These products in turn are exported from cells. PGD2 can also interact intracellularly with the \( \gamma \) form of the peroxisomal proliferator-activated receptor (15–17) to regulate adipocyte development.

These considerations suggest that LT, and glutathione conjugates formed intracellularly can potentially interact with a variety of intracellular proteins. To begin to understand the structural basis of these interactions we employed two photoaffinity ligands, S-(p-nitrobenzyl)glutathione-[125I]4-azidosalicylic acid (PNBG-[125I]ASA) and LTC4-[125I]4-azidosalicylic acid (LTC4-[125I]ASA), to identify the binding folds of proteins of membrane-associated proteins, which could recognize LTC4 and other glutathione conjugates. In studies with inside-out red blood cell vesicles, the major protein binding both probes was found to be glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Photoaffinity labeling of HL-60 cell cytosol also identified GAPDH as a protein binding LTC4.

Isolation and sequencing of iodinated peptides from the erythrocyte membrane protein and V8 protease digestion of labeled, homogeneous GAPDH indicated that both probes bound to the NAD\(^-\) binding pocket, or Rossmann fold (18, 19). The data suggest that GAPDH may also function as a not previously recognized class of proteins which bind LTC4 and glutathione conjugates.

**EXPERIMENTAL PROCEDURES**

**Reagents**—ATP, AMP, AMP-PCP, \( \beta \)-NAD\(^-\), S-(p-nitrobenzyl)glutathione, glyceraldehyde-3-phosphate, t-cysteine, Ponceau S, Trizma base, 2-mercaptoethanol, concanavalin A-agarose, Coomassie Brilliant Blue R-250, and GAPDH from human erythrocytes, Dulbecco’s minimal Eagle’s medium, and fetal bovine serum were purchased from Sigma. MgCl2·6H2O, NaOH, NH4HCO3, methanol, acetic acid, SDS, glycerol, sodium arsenate (Fisher), 6% dextrose 70 in 0.9% sodium chloride (Kendall-McGraw), N-hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA) (Pierce), LTC4 (BioMol), V8 protease (sequence grade), ultrapure glyceraldehyde-3-phosphate dehydrogenase; RP-HPLC, reverse phase-high performance liquid chromatography; PBS, phosphate-buffered saline; Tricine, N-tris(hydroxymethyl)methylglycine.
sucrose, β-octyl glucoside, protein G-Sepharose (Boehringer Mannheim), lysyl endopeptidase (Wako), dibasic sodium phosphate, and sodium pyrophosphate (Malinkrodt) were purchased. Mini Protein II Ready gels (Tri-glycine, 10%, and Tri-Tricine, 16.5%), kalcide/polyacrylamide standards, precasted low molecular weight standards, Silvented by Dr. Michael Silverstein, Temple University) was purchased from the spectrophotometrically by following the reduction of NAD+ at 340 nm (24). The assay contained 30 mM sodium pyrophosphate, 12 mM sodium arsenate, and 4 mM cysteine, pH 8.4. GAPDH (0.4 μg) was incubated for 20 min at 37 °C with the respective photoaffinity ligand, followed by addition of 400 μl of reaction buffer (Bio-Rad), Solvable tissue solubilizer, Fornax 986a, scintillation mixture was preincubated on ice or at 37 °C for 1 min. Uptake was initiated by the addition of 150 μM galecraldehyde-3-phosphate in a final reaction volume of 1 ml at 25 °C pH 8.4. All points were determined in duplicate at 30 s for calculations of initial velocity (V0) using a Beckman DU-64 spectrophotometer by measuring the change in absorbance of a mixture of 90 μM BAPTA and 0.001% bromphenol blue. The mixture was then poured into a 1.5-ml Eppendorf tube with the cap removed, chilled on ice for 10 min, and then exposed to long wave UV light (360 nm) for 7 min. The mixture was centrifuged at 14,000 × g for the supernatant discarded. The membranes were then solubilized in 100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 2% mercaptoethanol, and 0.001% bromphenol blue. Proteins were then separated by 10% SDS-PAGE using Bio-Rad Tricine gel in situ with 1% Coomassie Brilliant Blue or silver staining. Photoaffinity-labeled proteins were subsequently identified by autoradiography. Experiments labeling HL-60 with [125I]ASA were performed identically except that 250 μg of protein were used.

**Electroblotting and Sequencing**—Photoaffinity-labeled erythrocyte membrane proteins were separated by 10% SDS-PAGE and electroblotted to PVDF membranes in 25 mM Tris, 192 mM glycine buffer, pH 8.3, 20% methanol, in a Bio-Rad mini blotting apparatus at 100 V (constant) for 1 h. The membranes were stained with a solution of 0.5% Ponceau S in 1% acetic acid for 5 min and then destained with 1% acetic acid until the background was minimal, and then evaluated by autoradiography. Approximately 16 μg of the photoaffinity-labeled 38-kDa protein was transferred to PVDF membranes. The membrane containing the protein was cut out and the protein digested in situ with lysyl endopeptidase at the Whitehead Protein Sequencing Facility. The peptide fragments were separated by RP-HPLC (HP1090M) using a Vydac 2.1 × 250-mm C18 column and fractions collected by hand. Radioabeled peaks were identified by γ counting of each fraction and were analyzed on a gas phase microsequencer (model 475, Applied Biosystems). Proteins at the Whitehead Protein Sequencing Facility. Peptide searches were made using the BLAST program.

**HL-60 Cells and Preparation of Subcellular Fractions**—HL-60 cells were cutured to a density of 10⁹/ml in Dulbecco’s minimal Eagle’s medium with 10% fetal bovine serum. Cells were then collected by centrifugation at 1000 × g for 5 min and then washed three times in PBS. They were then resuspended at a concentration of 10⁹/ml in either 0.25% sucrose, 10 mM Tris HCl, 2 mM EDTA with 5 μM leupeptin, 1 μg/ml antipain, 7 μg/ml pepstatin, 5 μg/ml pepsin, or in 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 5 μg/ml leupeptin, 1 μg/ml antipain, 7 μg/ml pepstatin, 1 μg/ml EDTA for photoaffinity labeling. For Western blotting the cells were disrupted to greater than 90% in a Potter-Elvehjem homogenizer and then centrifuged first at 800 × g to remove unbroken cells and debris. The post-nuclear supernatant was centrifuged at 12,000 × g for 10 min, and the resulting supernatant was centrifuged at 100,000 × g for 1 h. The resulting pellet was resuspended in 1 ml of 250 mM sucrose, 10 mM Tris HCl, pH 7.4, and then repeatedly aspirated through a 27-gauge needle to disperse it. For photoaffinity labeling the cells were disrupted in a sonicating water bath and then centrifuged as above. Microsomes were resuspended in labeling buffer.

**Membrane Vesicle Transport**—LTC, and other glutathione conjugates are transported across membrane vesicles in an ATP-, Mg²⁺-, and temperature-dependent manner (10, 11, 21, 22). To determine the active transport of photoaffinity ligands, 6 pmol of PNBB-

**GAPDH Assays**—Glyceraldehyde-3-phosphate dehydrogenase was assayed spectrophotometrically by following the reduction of NAD+ at 340 nm (24). The assay contained 30 mM sodium pyrophosphate, 12 μM sodium arsenate, and 4 mM cysteine, pH 8.4. GAPDH (0.4 μg) was incubated for 20 min at 37 °C with the respective photoaffinity ligand, followed by addition of 400 μl of reaction buffer (Bio-Rad), Solvable tissue solubilizer, Fornax 986a, scintillation mixture was preincubated on ice or at 37 °C for 1 min. Uptake was initiated by the addition of 150 μM galecraldehyde-3-phosphate in a final reaction volume of 1 ml at 25 °C pH 8.4. All points were determined in duplicate at 30 s for calculations of initial velocity (V0) using a Beckman DU-64 spectrophotometer by measuring the change in absorbance of a mixture of 90 μM BAPTA and 0.001% bromphenol blue. The mixture was then poured into a 1.5-ml Eppendorf tube with the cap removed, chilled on ice for 10 min, and then exposed to long wave UV light (360 nm) for 7 min. The mixture was centrifuged at 14,000 × g for the supernatant discarded. The membranes were then solubilized in 100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 2% mercaptoethanol, and 0.001% bromphenol blue. Proteins were then separated by 10% SDS-PAGE using Bio-Rad Tricine gel in situ with 1% Coomassie Brilliant Blue or silver staining. Photoaffinity-labeled proteins were subsequently identified by autoradiography. Experiments labeling HL-60 with [125I]ASA were performed identically except that 250 μg of protein were used.
with Coomassie Brilliant Blue. Photoaffinity-labeled peptides and proteins were then identified by autoradiography.

\[ ^{3}H \text{LTC}_{4} \text{ Labeling, Gel Elution, and Counting—} \]

\[ ^{3}H \text{LTC}_{4} (6.5 \text{ kBq, } 20 \text{ nm}) \text{ was incubated with } 4 \mu \text{g of erythrocyte GAPDH in } 0.5 \text{ mM Tris, pH 7.5, for 10 min at 37 }^\circ \text{C, frozen in liquid nitrogen, and irradiated at 360 nm for 7 min (11). The labeled protein was mixed with SDS loading buffer, heated for 5 min at 95 }^\circ \text{C, analyzed by } 10\% \text{ SDS-PAGE, and stained with Coomassie Brilliant Blue. To locate the peptide incorporating the radioactivity, stained bands were excised and incubated with 0.5 ml of deionized water and 0.5 ml of Solvable for 3 h at 50 }^\circ \text{C, mixed with Formula 989 scintillation mixture, and then radioactivity quantitated by scintillation counting in a Packard 1500 Tri-Carb liquid scintillation counter.} \]

RESULTS

To determine the glutathione conjugate binding sites of proteins that regulate trafficking of LTC\(_4\) and other glutathione conjugates, we identified membrane proteins that were photoaffinity-labeled with both of the probes shown in Fig. 1, and also with 8-azido-[\(\alpha\)]\(^{32}\text{P}\)ATP. The latter was chosen to identify the nucleotide binding site of potential ATP-binding cassette carrier proteins. The membrane initially chosen for these studies and to test the probes was human erythrocyte inside out vesicles. Erythrocyte membranes export glutathione conjugates (21, 22), contain the multidrug resistance-associated protein (25), are simple in composition, and are available in large quantities. We initially determined whether the photoaffinity ligands were actively transported by erythrocyte membranes. The uptake of PNBG-[\(^{125}\text{I}\)]ASA and LTC\(_4\)-[\(^{125}\text{I}\)]ASA both increased over a period of 1–5 min in the presence of ATP and then leveled off over the next 25 min (data not shown). In the absence of ATP, or Mg\(^{2+}\), or when AMP-PCP or AMP was substituted for ATP no uptake was observed. When 3 pmol of PNBG-[\(^{125}\text{I}\)]ASA was used as a substrate, 157 fmol (mean, \(n = 2\)) was taken up at 1 min, whereas in the absence of ATP only 43.5 fmol was associated with the vesicles. When 6 pmol of LTC\(_4\)-[\(^{125}\text{I}\)]ASA was added in a reaction, 194 fmol was taken up at 1 min, whereas 36 fmol was associated with vesicles in the absence of ATP. These data indicated that these ligands were actively imported by erythrocyte membrane vesicles.

Erythrocyte membranes were then incubated on ice with PNBG-[\(^{125}\text{I}\)]ASA or LTC\(_4\)-[\(^{125}\text{I}\)]ASA, photolyzed with UV light, separated by SDS-PAGE, stained with Coomassie Brilliant Blue, and analyzed by autoradiography (Fig. 2A). One major band of 38 kDa was observed to be photoaffinity-labeled with LTC\(_4\)-[\(^{125}\text{I}\)]ASA (lane 1) or PNBG-[\(^{125}\text{I}\)]ASA (lane 2). A minor band was observed at 190 kDa under both circumstances, and at 80 kDa using LTC\(_4\)-[\(^{125}\text{I}\)]ASA as the probe. Labeling with 8-azido-[\(\alpha\)]\(^{32}\text{P}\)ATP showed multiple bands (Fig. 2B, lane 1), but included one at 38 kDa. Lane 2 shows that a single band is identified in these membranes by labeling with PNBG-[\(^{125}\text{I}\)]ASA.

To identify the prominent 38-kDa protein, erythrocyte membrane vesicles were labeled with PNBG-[\(^{125}\text{I}\)]ASA and then electroblotted to PVDF membranes. After staining with Ponceau S, \(\sim 16 \mu\text{g of protein was digested in situ with lysyl endopeptidase and the resulting peptides separated by RP-HPLC. Fractions corresponding to OD peaks were collected by hand and then analyzed by } \gamma \text{ counting. Two fractions (54 and 56) were found to contain radioactivity. Sequencing was able to identify the 11 N-terminal amino acids of the peptide collected in peak 54. When analyzed by the BLAST program, these amino acids could be aligned with amino acids 119–129 of human liver GAPDH as shown in Table 1.}

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TABLE I
Comparison of the amino acid sequences of iodinated peptides with GAPDHs

|          | GAPDH (liver) | Peak 54 | GAPDH (muscle) | Amino acid no. |
|----------|--------------|---------|----------------|----------------|
| A.       |              |         |                |                |
|          | V I I S A P S A D A P | IV I T A P E D D A L | I V I S A P S A D A P | 119 120 121 122 123 124 125 126 127 128 129 |
| B.       |              |         |                |                |
|          | V I I S A P S A D A P | IV I T A P E D D A L | I V I S A P S A D A P | 67 68 69 70 71 72 73 74 75 76 77  |
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FIG. 1. Structure of iodinated photoaffinity ligands. A, S-\([\rho\text{-nitrobenzyl}]\)glutathione-[\(^{125}\text{I}\)]ASA. B, leukotriene \(\text{C}_4\)-[\(^{125}\text{I}\)]ASA.

FIG. 2. Photoaffinity labeling of erythrocyte membrane vesicles. A, 50 \(\mu\text{g of erythrocyte vesicles and either 1.0 pmol of LTC}_4-\[^{125}\text{I}\]ASA (lane 1) or 0.4 pmol of PNBG-[\(^{125}\text{I}\)]ASA (lane 2) were mixed in 250 \(\mu\text{l of 0.5 mM Tris, pH 7.5, and chilled for 10 min at 0 }^\circ\text{C. The mixture was then irradiated at 360 nm for 7 min. After centrifugation at 14,000 } \times \text{ g, the pellet was dissolved in SDS loading buffer and analyzed by 10\% SDS-PAGE and autoradiography. B, 50 } \mu\text{g of erythrocyte vesicles was photoaffinity-labeled with either 75 pmol of 8-azido-\([\alpha\text{-}\text{ATP}}\) showed multiple bands (Fig. 2B, lane 1), but included one at 38 kDa. Lane 2 shows that a single band is identified in these membranes by labeling with PNBG-[\(^{125}\text{I}\)]ASA.
I, with an identity of 7/11 residues. When the peptide in Peak 56 was analyzed in the same manner, it could be aligned with an identity of 11/11 amino acids for amino acids 67 to 77 of liver GAPDH (Table I). Both peptides are contained in the NAD$^+$ binding region of the protein (18, 19).

To further confirm that the nucleotide binding site was photoaffinity-labeled, 5 μg of purified GAPDH were photoaffinity-labeled with PNBG-[125I]ASA in the presence of increasing concentrations of NAD$^+$ or ATP (Fig. 3). PNBG-[125I]ASA photolabeling was inhibited ~50% by 10 μM ATP and completely at a concentration of 100 μM. One hundred μM NAD$^+$ inhibited labeling by this compound by greater than 50%, and 10 μM NAD$^+$ almost completely inhibited labeling. Photoaffinity labeling of GAPDH by LTC$_4$-[125I]ASA was inhibited by ~50% with 1 μM ATP and almost completely at 10 μM ATP. Photoaffinity labeling with this probe was essentially abolished at 10 μM and 100 μM NAD$^+$.

To confirm that glutahtione conjugates that were not derivatized as photoaffinity labels interacted with the active site of GAPDH, we determined the $K_i$ for S-(p-nitrobenzyl)glutathione of GAPDH enzymatic activity. Fig. 4 shows the results of one of two experiments. For two experiments the $K_i$ was found to be 379 μM ± 6 (mean ± S.D., n = 2).

Because the peptide sequence of the 38-kDa protein was obtained with membranes labeled with the PNBG-[125I]ASA probe, we utilized the ability of V8 protease to cleave GAPDH into its NAD$^+$ and glyceraldehyde-3-phosphate binding domains to provide additional confirmation that LTC$_4$-[125I]ASA bound to the Rossmann fold. Four micrograms of enzyme were photoaffinity-labeled with either PNBG-[125I]ASA or LTC$_4$-[125I]ASA and then digested with 4 μg of V8 protease for 2 h at 37 °C. Digested (lanes 1 and 3) and undigested (lanes 2 and 4) GAPDH were separated by 16.5% Tris/Tricine-PAGE, and analyzed by either silver staining (A) and autoradiography (B).

FIG. 3. Inhibition of photoaffinity labeling of GAPDH. Five μg of human erythrocyte GAPDH was chilled at 0 °C for 10 min with the indicated concentrations of NAD$^+$ and ATP prior to photoaffinity labeling. The reaction was then mixed with SDS loading buffer and analyzed by SDS-PAGE and autoradiography.

FIG. 4. Inhibition of GAPDH activity. Secondary plot of slopes versus S-(p-nitrobenzyl)glutathione concentration used to determine the $K_i$ for this compound by greater than 50%, and 10 μM concentrations of NAD$^+$ indicated concentrations of NAD$^+$ of human erythrocyte GAPDH was chilled at 0 °C for 10 min with the active site of GAPDH, we determined the $K_i$ for S-(p-nitrobenzyl)glutathione concentration used to determine S-(p-nitrobenzyl)glutathione.

FIG. 5. PNBG-[125I]ASA and LTC$_4$-[125I]ASA bind to the Rossmann fold of GAPDH. Four μg of GAPDH was photoaffinity-labeled with either PNBG-[125I]ASA or LTC$_4$-[125I]ASA and then digested with 4 μg of V8 protease for 2 h at 37 °C. Digested (lanes 1 and 3) and undigested (lanes 2 and 4) GAPDH were separated by 16.5% Tris/Tricine-PAGE, and analyzed by either silver staining (A) and autoradiography (B).

FIG. 6. Photoaffinity labeling and identification of GAPDH in HL-60 cytosol. Two μg of GAPDH (lane 1) or 250 μg of HL-60 cytosol (lanes 2–4) were photoaffinity-labeled with LTC$_4$-[125I]ASA and then analyzed by SDS-PAGE with autoradiography. Lanes 1 and 2 were analyzed directly and lanes 3 and 4 after immunoprecipitation with either anti-GAPDH (lane 3) or anti-Rb(C36) (lane 4).
the radioactivity was distributed identically to that of the photoaffinity ligands, indicating that a natural ligand bound to the same site as the photoaffinity label (data not shown).

We next determined whether GAPDH in nucleated cells could interact with LTC$_4$-$[^{125}I]$ASA. In initial experiments, HL-60 cell fractions were analyzed by Western blotting to determine the distribution of GAPDH. Small amounts were found in the membrane fraction, and greater than 96% in the cytosol. Cytosol was then dialyzed against labeling buffer, labeled with LTC$_4$-$[^{125}I]$ASA, and then analyzed by SDS-PAGE and autoradiography. Two bands were identified, one with the identical size to GAPDH at 38 kDa and a second at ~27 kDa (Fig. 6). To confirm the identity of the 38-kDa protein, labeled cytosol was precipitated with antibody to GAPDH. A single band was found at 38 kDa (Fig. 6C), which was not seen with control antibody. Although GAPDH was found in the membrane fraction, this could not be successfully labeled. In addition, this small fraction could only be dissociated by the use of detergent, unlike the erythrocyte enzyme, which is released by salt (26).

**DISCUSSION**

Photoaffinity labeling of inner erythrocyte membranes by PNBG-$[^{125}I]$ASA or LTC$_4$-$[^{125}I]$ASA (Fig. 1) showed one predominant protein at 38 kDa and a second less intense band at 190 kDa (Fig. 2A). When analyzed by one-dimensional electrophoresis, the predominant 38-kDa protein appeared to also be labeled with 8-azido-$[^{32}P]$ATP (Fig. 2B). The strategy of labeling with two different probes was employed to identify members of the ATP-binding cassette family. However, carrier proteins that move midsize molecules contain at least six transmembrane domains and are at least 60 kDa in size (27). The size of 38 kDa was considered too small for such a protein. Because of its prominence, the 38-kDa band photolabeled with PNBG-$[^{125}I]$ASA was sequenced directly, with the assumption that even if there were several co-migrating proteins, labeled peptides could be isolated after digestion and sequenced. This approach proved viable in that only two peptides were identified after trypsin endopeptidase digestion and isolation by RP-HPLC. Peak 56 had 100% identity to liver GAPDH, whereas a single band was found at 38 kDa (Fig. 6C), which was not seen with control antibody. Although GAPDH was found in the membrane fraction, this could not be successfully labeled. In addition, this small fraction could only be dissociated by the use of detergent, unlike the erythrocyte enzyme, which is released by salt (26).

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