Parathyroid Hormone-dependent Degradation of Type II Na\(^+\)/Pi Cotransporters*

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Parathyroid hormone (PTH) inhibits proximal tubular brush border membrane Na\(^+\)/Pi cotransport activity; this decrease in the transport activity was found to be associated with a decrease in type II Na\(^+\)/Pi cotransporter protein content in rat brush border membranes. In the present study we investigated the PTH-dependent regulation of the type II Na\(^+\)/Pi cotransporter in opossum kidney cells, a previously established model to study cellular mechanisms involved in the regulation of proximal tubular Na\(^+\)/Pi cotransport. We transfected opossum kidney cells with a cDNA coding for NaPi-2 (rat renal type II Na\(^+\)/Pi cotransporter). This allowed the study of PTH-dependent regulation of the transfected NaPi-2 and of the corresponding intrinsic cotransporter (NaPi-4). The results show (i) that the intrinsic and the transfected cotransporters are functionally (transport) and morphologically (immunofluorescence) localized at the apical membrane, (ii) that the intrinsic as well as the transfected Na\(^+\)/Pi cotransport activities are inhibited by PTH, (iii) that PTH leads to a retrieval of both cotransporters from the apical membrane, (iv) that both cotransporters are rapidly degraded in response to PTH, and (v) that the reappearance/recovery of type II Na\(^+\)/Pi cotransporter protein and function from PTH inhibition requires de novo protein synthesis. These results document that PTH leads to a removal of type II Na\(^+\)/Pi cotransporters from the apical membrane and to their subsequent degradation.

In the present study we investigated the PTH-mediated regulation of the type II Na\(^+\)/Pi cotransporter in opossum cells (OK cells); these cells have recently been shown to contain such a cotransporter (NaPi-4; Ref. 8). The validity of the opossum kidney cell model to study proximal tubular Na\(^+\)/Pi cotransport and its regulation has been established (18–23). With respect to PTH-dependent control of Na\(^+\)/Pi cotransport activity, we have reported that the recovery from the PTH-mediated inhibition of Na\(^+\)/Pi cotransport in OK cells is dependent on de novo protein synthesis. This latter observation led to the hypothesis that PTH might lead to the retrieval and degradation of the transporter (27).

The aims of the present study were 2-fold: (i) to study cellular/molecular mechanisms involved in PTH-dependent control of type II Na\(^+\)/Pi cotransporters, (ii) to create by transfection an in vitro model that also permits the study of the PTH control of the rat type II Na\(^+\)/Pi cotransporter. Obviously, the latter approach would then offer a tool to characterize the molecular determinants involved in such regulations. A prerequisite for this approach was the availability of antisera, permitting a distinction between intrinsic (NaPi-4) and transfected (NaPi-2) cotransporters.

The results obtained show that both intrinsic (NaPi-4) and transfected type II Na\(^+\)/Pi cotransporters (NaPi-2, rat) are functionally (transport) and morphologically (immunofluorescence) located at the apical cell surface, are functionally inhibited in response to PTH addition, are retrieved in a PTH-dependent manner from the apical cell surface (immunofluorescence), and are subsequently degraded (Western blots). These data document that PTH control of the type II Na\(^+\)/Pi cotransporters involves a step of membrane retrieval and degradation. Furthermore, the OK cell system should represent the ideal in vitro model to dissect the cellular/molecular mechanisms participating in regulation of this proximal tubular transport function, which is crucially involved in overall Pi homeostasis.

EXPERIMENTAL PROCEDURES

Vectors—The generation of the vectors used (pLKneo and NaPi-2/pLKneo) has been described previously (24, 25). Both vectors code for a geneticin (G418) resistance under a SV40 promoter. In addition, the vector NaPi-2/pLKneo contains a cDNA coding for the rat renal type II Na\(^+\)/Pi cotransporter (NaPi-2) under a dexamethasone-inducible promoter.

Cell Cultures and Transfections—All cell culture supplies were obtained from Life Technologies, Inc. (Basel, Switzerland). Opossum kidney cells (clone 3B/2) were maintained in Dulbecco's modified Eagle’s medium/Ham's F-12 medium (1:1) supplemented with 10% fetal calf serum, 22 mM NaHCO\(_3\), 20 mM Hepes, 2 mM d-glutamine, 50 IU/ml penicillin, and 50 µg/ml streptomycin in a humidified atmosphere of 5% CO\(_2\), 95% air at 37 °C. Monolayers on permeable filter supports were grown on Millicell-CM filter inserts (Millipore; 12-mm diameter, 0.45-µm pore size) coated with a very thin film of rat tail collagen (R type, 0.5 mg/ml in 50% DMSO).
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ethanol; Serva, Basel, Switzerland). Cells were seeded at approximately 1–2 × 10⁵ cells/filter and were re-fed with fresh medium every 12 h until reaching confluency 24 h after seeding. Transport studies were commenced 36 h after seeding. The tightness of confluent cell monolayers has been measured by resistance measurements as described previously (22).

For transfection, cells grown to a confluency of approximately 60% in 35 mm dishes (Nunc) were incubated for 16 h with 20 μl of a 1:1 mixture of water and Lipofectin (Life Technologies) containing 10 μg of either NaPi-2/pLkNeo or the same amount of empty vector pLkNeo. Afterward, cells were trypsinized, split at a ratio of 1:30, and grown in 150 mm dishes (Nunc) in medium containing, in addition to the extra-active geneticin. After 1–2 weeks, colonies of geneticin-resistant cells were isolated by ring cloning, expanded, and analyzed for the expression of the NaPi-2 protein by immunoblotting (see below). For experimental purposes, transfected cells were used within 10 passages. Dexamethasone induction was performed by an incubation with 1 μM dexamethasone (Sigma) for 20 h (10,000-fold stock, made in ethanol).

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—Cells grown to confluency in 10-cm Petri dishes were incubated either with or without dexamethasone (1 μM) for 20 h and washed twice with TBS (0.9% NaCl, 10 mM Tris-HCl, pH 7.4). 15 ml of TBS containing 4 mM EDTA and 1 mM phenylmethylsulfonyl fluoride was added, and the cells were scrapped off the dish. The scraped cells were homogenized 5 times by liquid scintillation and, by liquid scintillation and a range needed to obtain a single clone (antibody dilution, 1/4000). The intrinsic NaPi-4 protein was detected as nmol of P, taken up/mg of total cellular protein, which was determined by the Bio-Rad protein assay.

For transport assays on permeant filter supports, growth medium was aspirated, and both sides of the monolayer were gently rinsed twice in substrate-free uptake solution. Filter insert monolayers were then placed in a 24-well culture plate (Nunc) for uptake measurements. Substrate-free uptake solution (500 μM) was added to the appropriate filter insert compartment, and N-methyl-D-glucamine-HCl uptake solution was added to the opposite compartment. Uptake was initiated by mixing 50 μl of the same uptake solution containing an 11-fold concentration of the desired radiolabeled substrate to the uptake solution already present in the filter insert compartment. Uptake was stopped at specific times (10, 20, 30, 60, and 120 min) by washing the monolayers with PBS containing 0.5 mM MgCl₂ and 1 mM CaCl₂, cells were fixed for 10 min with 4% paraformaldehyde, washed three times with PBS, incubated 10 min with 20 nmol of Pi taken up/mg of total cellular protein, which was determined by the Bio-Rad protein assay.

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Expression of the NaPi-2 protein in transfected OK cells, and expression of the intrinsic NaPi-4 protein in untransfected OK cells. NaPi-2-transfected (A) and untransfected OK cells (B) were grown to confluency on plastic Petri dishes and, where indicated (DEX, +), were exposed to 1 \(\mu\)M dexamethasone for 20 h. Dexamethasone-induced NaPi-2-transfected OK cells showed an approximately 2-fold stimulation of the Na\(^{+}/P\) cotransport activity, whereas dexamethasone had no significant effect on the intrinsic Na\(^{+}/P\), cotransport in empty vector-transfected and untransfected OK cells.

Expression of the type II transporter was also analyzed by immunofluorescence. Immunofluorescence pictures obtained by confocal microscopy showed that both the intrinsic NaPi-4 (Fig. 3A) and the transfected NaPi-2 (Fig. 3B) are localized at the apical membrane within distinct clusters of a diameter of about 1–2 \(\mu\)m, whereas no type II Na\(^{+}/P\) cotransporter-specific staining was seen at the basolateral membrane. The immunohistochemical staining of the transfected NaPi-2 as well as of the intrinsic NaPi-4 could be specifically abolished by the corresponding antigenic peptide (100 \(\mu\)g/ml; data not shown). In Figs. 3A and 3B, the parallel staining for \(\beta\)-actin (a component of the microvillar cytoskeleton) is shown. It is apparent that NaPi-4-specific (Fig. 3A) and NaPi-2-specific (Fig. 3B) staining on the apical cell surface coincides with the \(\beta\)-actin staining. In addition to the above apical staining, \(\beta\)-actin is also present at the basolateral cell surfaces (Fig. 3A and 3B). Visualization of the apical surface of OK cells by scanning electron microscopy showed that microvilli are expressed at the apical surface, forming distinct clusters (Fig. 3C); the diameter of the clusters

![Figure 1](image1.png)

**Fig. 1.** Dexamethasone-induced expression of Na\(^{+}/P\) cotransport in NaPi-2-transfected OK cells. OK cells (NaPi-2-transfected, empty vector-transfected, and untransfected OK cells) were grown to confluency on plastic Petri dishes and, where indicated (DEX, +), were exposed to 1 \(\mu\)M dexamethasone for 20 h. Dexamethasone-induced NaPi-2-transfected OK cells showed an approximately 2-fold stimulation of the Na\(^{+}/P\) cotransport activity, whereas dexamethasone had no significant effect on the intrinsic Na\(^{+}/P\), cotransport in empty vector-transfected and untransfected OK cells.

![Figure 2](image2.png)

**Fig. 2.** Expression of the NaPi-2 protein in transfected OK cells, and expression of the intrinsic NaPi-4 protein in untransfected OK cells. NaPi-2-transfected (A) and untransfected OK cells (B) were grown to confluency on plastic Petri dishes and, where indicated (DEX, +), were treated with dexamethasone (1 \(\mu\)M, 20 h). Crude membrane preparations were analyzed by immunoblotting using an anti-NaPi-2 antiserum (A) or an anti-NaPi-4 antiserum (B). Incubation with primary antibody was performed in the absence (Peptide, −) or in the presence (Peptide, +) of the corresponding antigenic peptide (peptide protection). The staining seen above the 116-kDa region in the Fig. 2A is unspecific (see text).

![Figure 3](image3.png)

**Fig. 3.** The intrinsic NaPi-4 protein and the transfected NaPi-2 protein are expressed at the apical membrane of OK cells within microvilli. NaPi-2-transfected and untransfected OK cells were grown to confluency on glass coverslips and further processed for immunofluorescence (A and B). Each set of pictures obtained by confocal microscopy contains an apically located focal plane (xy-plane) and cross-sections corresponding to xz and yz planes. White lines in the xy plane indicate the different sections along the z axis. White lines in the xz and yz planes indicate the location of the xy plane. A and B show immunohistochemical double stainings of \(\beta\)-actin and NaPi-4 in untransfected OK cells (A) and \(\beta\)-actin and NaPi-2 in dexamethasone-induced NaPi-2-transfected OK cells (B). It is seen that \(\beta\)-actin and NaPi-4 and \(\beta\)-actin and NaPi-2 colocalize within distinct clusters at the apical membrane. Scanning electron microscopy demonstrates the presence of clustered microvilli at the apical surface of OK cells (C). The diameter of these clustered microvilli corresponds well with the diameter of the distinct clusters at the apical membrane seen in the immunofluorescence pictures in Fig. 3, A and B.

Expression of the type II transporter was also analyzed by immunofluorescence. Immunofluorescence pictures obtained by confocal microscopy showed that both the intrinsic NaPi-4 (Fig. 3A) and the transfected NaPi-2 (Fig. 3B) are localized at the apical membrane within distinct clusters of a diameter of about 1–2 \(\mu\)m, whereas no type II Na\(^{+}/P\) cotransporter-specific staining was seen at the basolateral membrane. The immunohistochemical staining of the transfected NaPi-2 as well as of the intrinsic NaPi-4 could be specifically abolished by the corresponding antigenic peptide (100 \(\mu\)g/ml; data not shown). In Figs. 3A and 3B, the parallel staining for \(\beta\)-actin (a component of the microvillar cytoskeleton) is shown. It is apparent that NaPi-4-specific (Fig. 3A) and NaPi-2-specific (Fig. 3B) staining on the apical cell surface coincides with the \(\beta\)-actin staining. In addition to the above apical staining, \(\beta\)-actin is also present at the basolateral cell surfaces (Fig. 3A and 3B). Visualization of the apical surface of OK cells by scanning electron microscopy showed that microvilli are expressed at the apical surface, forming distinct clusters (Fig. 3C); the diameter of the clusters...
corresponds well with the diameter of the clusters seen by immunofluorescence double staining for β-actin and the corresponding type II Na⁺/Pᵢ cotransporter. Therefore the data given in Fig. 3 (A–C) documents that intrinsic and transfected type II Na⁺/Pᵢ cotransporters are predominantly expressed at the apical cell surface (most likely within microvilli).

**PTH-dependent Regulation of Type II Na⁺/Pᵢ Cotransporters**—Fig. 4 summarizes the effect of PTH (10⁻⁸ M; 4 h) on the Na⁺-dependent Pᵢ transport in NaPᵢ-2-transfected OK cells as well as in untransfected OK cells. PTH inhibited the Na⁺-dependent Pᵢ transport activity in control cells by about 60%. A similar inhibitory effect of PTH was observed in NaPᵢ-2-transfected cells that were not treated with dexamethasone. In cells expressing the NaPᵢ-2 transporter (induced by dexamethasone), PTH also inhibited the additionally expressed Na⁺/Pᵢ cotransport activity. Interestingly, the residual transport activity after PTH treatment was similar in all cells tested. This latter observation is in agreement with earlier studies (21, 27) demonstrating a PTH-insensitive Na⁺/Pᵢ cotransport activity in OK cells.

The effect of PTH on the type II Na⁺/Pᵢ cotransporter protein content was investigated by immunoblotting. Fig. 5 shows that incubating OK cells for increasing times with PTH (10⁻⁸ M) leads to a time-dependent decrease of NaPᵢ-4 and NaPᵢ-2 protein expressed in OK cells. We conclude that the whole protein was degraded due to PTH action and that both transfected (NaPᵢ-2) and intrinsic transporter (NaPᵢ-4) behave very similarly, i.e. are degraded. The finding that PTH leads to the degradation of the type II Na⁺/Pᵢ cotransporter is in agreement with our previous observation that the recovery of the Na⁺/Pᵢ cotransport after PTH-mediated inhibition is dependent on de novo protein synthesis (27, 28). To evaluate whether the recovery of the transport was due to de novo synthesis of type II Na⁺/Pᵢ cotransporter, cell monolayers were exposed to 10⁻⁸ M PTH for 4 h; PTH was removed, and cell monolayers were incubated with normal medium in the presence or absence of 20 μM cycloheximide for 2 or 4 h. The results presented in Fig. 6 show that the reappearance of the transporter is dependent on de novo protein synthesis. It is also seen that 4 h after PTH removal, the reappearance of the transporter is not completed. Parallel measurements of the Na⁺/Pᵢ cotransport showed that after 4 h of PTH removal, the recovery of the Na⁺/Pᵢ cotransport is also incomplete. This is in agreement with our previous observation that complete recovery of Na⁺/Pᵢ cotransport from PTH inhibition takes about 10–12 h (28).

The retrieval of the intrinsic (NaPᵢ-4) and the transfected cotransporter (NaPᵢ-2) upon addition of PTH could also be documented by immunofluorescence. Consistent with the data presented above (Figs. 4–6), we found that incubating OK cells with PTH (10⁻⁸ M) leads to a time-dependent decrease of NaPᵢ-4-dependent immunofluorescence and NaPᵢ-2-dependent immunofluorescence at the apical membrane of OK cells (data not shown). After 4 h of PTH treatment (10⁻⁸ M), the Na⁺/Pᵢ cotransporter type II-specific immunofluorescence staining in untransfected (Fig. 7A) and NaPᵢ-2-transfected OK cells (Fig. 7B) was virtually absent. Corresponding immunofluorescence pictures (double immunofluorescence) stained for β-actin clearly showed that under these conditions the microvilli were still present at the apical surface of OK cells. With the aim to detect the retrieved Na⁺/Pᵢ cotransporters within intracellular vesicles, we have treated OK cells for increasing times with PTH (0, 0.5, 1, 2, and 4 h; data not shown). As mentioned above, we observed a time-dependent decrease of the NaPᵢ-4-specific staining and NaPᵢ-2-specific staining at the apical surface. However, we were not able to detect a significant amount of NaPᵢ-4 protein or NaPᵢ-2 protein within intracellular vesicles at any time points (data not shown). As already suggested by the experiments presented in Fig. 5, a delay between a PTH-dependent membrane retrieval and intracellular degradation seems to be absent or minimal.
Previous studies on rats have suggested that inhibition of Na\textsuperscript{+}/P\textsubscript{i} cotransport in renal proximal tubules by PTH involves a retrieval of the type II Na\textsuperscript{+}/P\textsubscript{i} cotransporter (NaPi-2) from the brush border membrane (15). The recent cloning of the type II Na\textsuperscript{+}/P\textsubscript{i} cotransporter of OK cells (NaPi-2; Ref. 8) and the obvious limitations of an in vivo system to study molecular mechanisms prompted us to investigate the regulation of this transporter by PTH in OK cells. OK cells are a renal epithelial cell line that has been used successfully to investigate mechanisms involved in the control of proximal tubular Na\textsuperscript{+}/P\textsubscript{i} cotransport by PTH (21, 27). In the present study, we stably transfected OK cells with a cDNA coding for the rat type II Na\textsuperscript{+}/P\textsubscript{i} cotransporter (NaPi-2) and investigated whether similar mechanisms are involved in the PTH-mediated regulation of the intrinsic (NaPi-4) and the transfected transporters (NaPi-2).

On immunoblots, appearance of the transfected (NaPi-2) and the intrinsic (NaPi-4) cotransporter proteins resembled each other closely. The broad staining pattern (95–120 kDa) of these transporters likely represents different degrees of glycosylation, which for the NaPi-2 protein, has recently been demonstrated (29). The expression of the rat type II Na\textsuperscript{+}/P\textsubscript{i} cotransporter was also demonstrated by transport experiments. Dexamethasone-induced NaPi-2-transfected OK cells exhibited an approximately 2-fold-stimulated transport activity compared with uninduced NaPi-2-transfected or control cells (untransfected and empty vector-transfected). Immunofluorescence experiments demonstrated an exclusive apical localization for both Na\textsuperscript{+}/P\textsubscript{i} cotransporters (intrinsic and transfected), that, in the case of the intrinsic NaPi-4, is in agreement with earlier transport studies performed with OK cells grown on permeant filter supports (22). Furthermore, immunofluorescence demonstrated a concentration of the intrinsic as well as of the transfected Na\textsuperscript{+}/P\textsubscript{i} cotransporters within clustered microvillar structures. It is suggested that such a distinct concentration of the Na\textsuperscript{+}/P\textsubscript{i} cotransporters within the microvilli may be due to a yet unknown interaction of these transporters with components of the microvillar cytoskeleton. Clearly no immunostaining related to these transporters was detected in the basolateral membrane, suggesting a specific apical sorting mechanism for the type II Na\textsuperscript{+}/P\textsubscript{i} cotransporters in OK cells. In contrast, we recently demonstrated that the NaPi-2, when transfected into Madin-Darby canine kidney cells, is expressed to equal amounts at the apical and basolateral membrane (25).

The observed different sorting behavior of the same transporter in two different cell lines indicates that not only molecular determinants are decisive for a polarized sorting but that also the cellular context is of importance. This has also been documented for other proteins and cell systems. For example, it has been shown that aquaporin-2, when transfected into LLC-PK\textsubscript{1} cells, is, upon cAMP stimulation, inserted into the basolateral membrane rather than into the apical membrane as suggested from studies on native renal epithelia (30).

In previous studies it has been demonstrated that the intrinsic apical Na\textsuperscript{+}/P\textsubscript{i} cotransport activity in OK cells is inhibited by PTH (21, 27). In the present study we extended this observation by showing that the inhibition of the Na\textsuperscript{+}/P\textsubscript{i} cotransport by PTH in OK cells is paralleled by the disappearance of the apical, Na\textsuperscript{+}/P\textsubscript{i}, cotransporter-specific immunofluorescence staining within the microvilli. It is also shown that PTH leads to the degradation of the type II Na\textsuperscript{+}/P\textsubscript{i} cotransporter in OK cells. This finding is in agreement with our earlier observation demonstrating that the recovery of Na\textsuperscript{+}/P\textsubscript{i} cotransport activity from PTH inhibition in OK cells is dependent on de novo protein synthesis. Correspondingly, we found that the recovery of Na\textsuperscript{+}/P\textsubscript{i} cotransport activity from PTH inhibition was paral-
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...led on immunoblots by the reappearance of the type II Na\(^+\)/P\(^i\) cotransporter protein. In the presence of cycloheximide, there was neither a recovery of transport activity nor a reappearance of the specific transporter protein. As documented by immunoblots and by immunohistochemical studies presented in this paper, PTH leads to an almost complete retrieval and degradation of the type II Na\(^+\)/P\(^i\) cotransporter (Figs. 5 and 7). However, even after prolonged exposures to PTH, a "refractory" residual Na\(^+\)/P\(^i\) cotransport activity is observed (Fig. 4 and Ref. 21). This implies that this residual activity (~40% of total transport activity) is not related to the type II Na\(^+\)/P\(^i\) cotransporter but could be associated with, for example, the type I transporter. Such conclusions are also valid for the Na\(^+\)/P\(^i\) cotransport activity in rat brush border membranes; 2 h of PTH treatment leads to a much higher reduction of the Na\(^+\)/P\(^i\) protein compared with the reduction in Na\(^+\)/P\(^i\) cotransport activity (15).

The present study clearly indicates that the regulation of the type II Na\(^+\)/P\(^i\) cotransporter in renal proximal tubules and in OK cells is very similar. One apparent difference exists: after PTH treatment of OK cells for various lengths (0.5, 1, 2, and 4 h) we were not able to detect a significant transient intracellular staining specific for the type II Na\(^+\)/P\(^i\) cotransporter. In rat proximal tubules, the degradation of the Na\(^+\)/P\(^i\) protein seems to be delayed (compared with the OK cell system), permitting a visualization of an increased intracellular Na\(^+\)/P\(^i\) protein content after short (15 min to 1 h) but not after prolonged treatments with PTH (15). Despite this difference, the similarities in the regulation of this transporter by PTH in OK cells and in rat renal proximal tubules are evident. Furthermore, in this report we demonstrated that the transfected (Na\(^+\)/P\(^i\)-2) and intrinsic type II Na\(^+\)/P\(^i\) cotransporters (Na\(^+\)/P\(^i\)-4) are regulated in OK cells in the same way by PTH. OK cells are therefore a physiologically relevant in vitro system for the study of the regulation of the type II Na\(^+\)/P\(^i\) cotransporter type II.

They are a useful tool to dissect the molecular/cellular mechanisms involved in the PTH-mediated internalization and subsequent degradation of the transporter. Although it is tempting to assume a final breakdown of these Na\(^+\)/P\(^i\) cotransporters within lysosomes, the mechanisms involved in internalization, trafficking, and subsequent degradation are completely unknown.

In summary, the present study shows (i) that the intrinsic Na\(^+\)/P\(^i\)-2 and the transfected rat type II Na\(^+\)/P\(^i\) cotransporter Na\(^+\)/P\(^i\)-2 are functionally and morphologically localized at the apical membrane, (ii) that the intrinsic as well as the transfection-mediated Na\(^+\)/P\(^i\)-2 Na\(^+\)/P\(^i\) cotransport activities are inhibited by PTH, (iii) that PTH leads to the disappearance of both cotransporters from the apical membrane, and (iv) that both cotransporters are rapidly degraded in response to PTH. These results suggest that PTH leads to the endocytosis of the type II Na\(^+\)/P\(^i\) cotransporters from the apical membrane and to their subsequent degradation, thereby leading to the down-regulation of the Na\(^+\)/P\(^i\) cotransport.

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