Structure–activity analysis of synthetic α -thrombin-receptor-activating peptides

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α -Thrombin stimulates G-protein-coupled effectors leading to secretion and aggregation in human platelets, and to a mitogenic response in CCL39 hamster fibroblasts. α -Thrombin receptors can be activated by synthetic peptides corresponding to the receptor sequence starting with serine-42, at the proposed cleavage site. We have previously determined that the agonist domain of receptor-activating peptides resides within the five N-terminal residues [Vouret-Craviari, Van Obberghen-Schilling, Rasmussen, Pavirani, Lecocq and Pouyssegur (1992) *Mol. Biol. Cell.* **3**, 95–102], although the 7-residue peptide (SFLLRNP) corresponding to the hamster α -thrombin receptor was 10 times more potent than the 5-residue peptide for activation of human platelets. In the present study we have analysed the role of individual amino acids in receptor activation by using a series of modified hexa- or hepta-peptides derived from the human α -thrombin-receptor sequence. Cellular events examined here include phospholipase C activation, adenylyl cyclase inhibition and DNA synthesis stimulation in non-transformed CCL39 fibroblasts and a tumorigenic variant of that line (A71 cells).

Modification of the peptide sequence had similar functional consequence for each of the assays described, indicating that either a unique receptor or pharmacologically indistinguishable receptor subtypes activate distinct G-protein signalling pathways. Furthermore, we found that: (1) the N-terminal serine can be replaced by small or intermediately sized amino acids ($\pm$ hydroxyl groups) without loss of activity. However, its replacement by an aromatic side-chain or omission of the N-terminal amino group severely reduces activity. (2) An aromatic side-chain on the penultimate N-terminal residue appears to play a critical role since phenylalanine in this position can be substituted by tyrosine without complete loss of activity whereas an alanine in its place is not tolerated. (3) Deletion of the first, second or third N-terminal residue leads to a loss of activity, suggesting that a defined spacing of more than one structural component may be important for ligand–receptor interaction. Finally, we did not observe an antagonistic effect of the inactive peptides on phospholipase C activation or DNA synthesis induced by α -thrombin (1 nM) or SFLLRNP (3 μ M).

INTRODUCTION

The serine protease α -thrombin activates intracellular signalling pathways in a variety of cells via a plasma membrane substrate which has been cloned from human erythroleukemia (HEL) cells [1] and hamster fibroblasts [2]. This α -thrombin substrate, or receptor, is a member of the G-protein-coupled receptor superfamily that displays an α -thrombin cleavage site in the N-terminal extracellular domain. According to the current model, cleavage of the receptor by α -thrombin unmasks a new N-terminus which functions as a tethered ligand. Evidence for this proteolytic mechanism of activation is supported by the findings that a synthetic peptide corresponding to the N-terminal sequence of the cleaved receptor (residues Ser-42 to Phe-55) is able to stimulate aggregation of human platelets and Ca^{2+} release in *Xenopus* oocytes expressing the cloned human receptor [1]. Further, this 14-amino-acid peptide (SFLLRNPNDKYEPF) has been found to trigger several intracellular signalling events in human platelets including phospholipase C and phosphatidylinositol 3-kinase activation [3], adenylyl cyclase inhibition and phosphorylation of proteins on tyrosine residues [4,5].

Activation of non-platelet cellular responses also appears to be mediated by the cloned receptor, or a similar subtype. Peptides derived from the human or hamster α -thrombin receptor sequence are able to mimic α -thrombin action on G-protein-coupled effectors in fibroblasts [6] and HEL cells [7], to elevate intracellular Ca^{2+} and stimulate prostacyclin production in

human endothelial cells [8], and to cause neurite retraction in neuronal cells [9]. However, receptor-activating peptides are not as effective as α -thrombin at stimulating p44 mitogen-activated protein kinase or growth of CCL39 fibroblasts [6,10], suggesting the involvement of ancillary proteins or additional receptor-activated signalling pathways in these effects. In the light of the multifunctionality of α -thrombin, selective receptor antagonists would be useful pharmacological tools and potential therapeutic agents.

We have determined previously that only the five N-terminal residues of the hamster α -thrombin-receptor-activating peptide are required for full agonist activity in human platelets and CCL39 fibroblasts [6]. However, the 7-residue peptide (SFLLRNP) was found to be most effective in stimulating G-protein-coupled effectors in these cells. Similarly, Vassallo et al. observed that the human receptor hexa-peptide (SFLLRN) was five times more potent than the 14-residue agonist for platelet aggregation [5]. In the present study we have used a series of modified α -thrombin-receptor hexa- and hepta-peptides to assess the role of individual amino acids in receptor activation.

MATERIALS AND METHODS

Cells and culture conditions

Cells used in this study were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 7.5 % (v/v) fetal-calf serum (FCS), penicillin (50 units/ml) and streptomycin (50 μ g/ml). The

Abbreviations used: HEL, human erythroleukemia; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum; FGF, fibroblast growth factor; DTT, dithiothreitol; H21/F12 medium, 1:1 mixture of DMEM and Ham's F12 medium.

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A71 line was derived from CCL39 Chinese hamster lung fibroblasts (American Type Culture Collection) following mutagenesis and selection for growth in soft agar, as described [11]. Cell cultures were growth-arrested by serum removal for 24 h.

Materials

Pure human α -thrombin (3200 NIH units/mg) and human recombinant basic fibroblast growth factor (FGF) were generously provided by Drs. J. W. Fenton II (New York State Department of Health, Albany, NY, U.S.A.) and D. Gospodarowicz (University of California Medical Center, San Francisco, CA, U.S.A.) respectively. Synthetic α -thrombin-receptor peptides (> 95% pure) were purchased from NEOSYSTEM (Strasbourg, France). For assays of phospholipase C activation and DNA synthesis re-initiation, peptides were dissolved in Hepes-buffered (20 mM; pH 7.4) DMEM to a final concentration of 3 mM, except for the human MFLLRNP peptide, which was soluble at 1 mM. A 5-fold molar excess of dithiothreitol (DTT) was added to the CFLLRNP peptide, where indicated, and the effect of DTT in bioassays was monitored. *Myo*-[2-³H]inositol and [*methyl*-³H]thymidine were from Amersham (Les Ulis, France).

Activation of phospholipase C

Cells in 12-well plates were growth-arrested for 24 h in the presence of [³H]inositol (2 μ Ci/ml). Before the assay cells were rinsed twice with Hepes-buffered (20 mM; pH 7.4) DMEM then incubated in the same buffer containing 20 mM LiCl for 4 min before adding agonists. After the indicated times of stimulation with α -thrombin and/or peptides, the reaction was stopped with 10 mM ice-cold formic acid and inositol phosphates were separated by anion-exchange chromatography and counted by liquid-scintillation spectrophotometry as reported [12].

Inhibition of adenylyl cyclase

Inhibition of adenylyl cyclase activity by peptides in forskolin (50 μ M)-stimulated cells in the presence of 1 mM isobutylmethylxanthine (10 min incubation) was determined as previously described [6].

Re-initiation of DNA synthesis

Cells were grown to confluence in 24-well plates before serum deprivation for 24 h in a 1:1 mixture of DMEM and Ham's F12 medium (H21/F12) to permit entry into the G₀/G₁ phase of the cell cycle. Peptides and growth factors were added in fresh H21/F12 medium containing 1 μ Ci/ml [*m*-³H]thymidine (3 μ M thymidine). After 24 h, [*m*-³H]thymidine incorporation was stopped with 5% (w/v) trichloroacetic acid and trichloroacetic acid-precipitable radioactivity was determined in a liquid-scintillation counter.

RESULTS

The CCL39 hamster fibroblast line was chosen as a model system for structure-activity studies on α -thrombin-receptor-activating peptides since α -thrombin-activated signalling pathways have been well characterized in these cells (for reviews [13,14]). Peptide sequences used in this study and their corresponding positions in the human, or Chinese hamster, receptor are detailed in Table 1. We have previously determined that a 7-residue peptide corresponding to position Ser-42-Pro-48 of the hamster α -thrombin receptor was as effective as the 14-residue human receptor

peptide (Ser-42-Phe-55) at activating human platelets and initiating cellular responses in hamster fibroblasts [6]. Further, it has been shown that functional determinants reside in the N-terminus of the receptor peptide agonist since deletion or inversion of the two N-terminal residues totally suppresses activity [1]. Therefore, we have determined the activity of heptapeptides with N-terminal substitutions of residues in the first or second position, as well as peptide with deletions of the first, second or third N-terminal residue. Cellular responses examined here include the rapid activation of G-protein-coupled effectors, phospholipase C and adenylyl cyclase, and more long-term effects on DNA synthesis re-initiation in serum-deprived cells.

Phospholipase C activation

α -Thrombin-receptor-activating peptides, like α -thrombin, stimulate a phosphoinositide-specific phospholipase C and induce desensitization of the α -thrombin response. The sequences of human and hamster α -thrombin-activating heptapeptides differ by only one amino acid (a leucine to phenylalanine change in position 3). We found a similar potency of hamster and human peptides for phospholipase C activation in CCL39 hamster fibroblasts (EC₅₀ approx. 20 μ M) [6], suggesting that at least for phospholipase C stimulation this change has no functional consequence.

The peptides listed in Table 1 (30 μ M) were screened for their ability to stimulate phospholipase C activity during a 10 min

Table 1 Thrombin receptor peptides and their cellular effects

One-letter abbreviations for the amino-acid sequences of synthetic peptides used in this study, and their corresponding residues in the published human [1] or Chinese hamster [2] receptor sequences are indicated. The abbreviation prop represents propionic acid (desamino alanine). Residues printed in bold differ from those present in the published human thrombin receptor sequence. Acidic amino acids in the 'negative-charge cluster' are shaded. The effects of peptides (30 μ M; 10 min incubation) on phospholipase C (PLC) activation and inhibition of forskolin-stimulated adenylyl cyclase were examined in CCL39 cells. Relative effects of modified peptides compared with the 'wild-type' sequence are expressed as —, < 5%; +, $\leq$ 25%; ++, 25–75%; and + + +, $\geq$ 75%. EC₅₀ values are listed for stimulation of [*m*-³H]thymidine incorporation by receptor peptides in A71 cells; values were obtained from at least two independent experiments. The effects of peptide CFLLRNP were determined in the presence of DTT, as indicated in the Materials and methods section.

(Residues)		PLC activation	Adenylyl cyclase inhibition	DNA synthesis EC ₅₀ (μ M)
Human receptor sequence				
(42–55)	SFLLRNPNDKYGPF (S-14-F)	+++	+++	10 $\pm$ 2.5
(42–48)	SFLLRNP	+++	+++	50 $\pm$ 2.5
	TFLLRNP	+++	+++	75 $\pm$ 5
	AFLLRNP	+++	+++	75 $\pm$ 10
	CFLLRNP	+++	+++	60 $\pm$ 2.5
	MFLLRNP	+++	+++	60 $\pm$ 7.5
	prop FLLRNP	++	++	150 $\pm$ 25
	YFLLRNP	+	+	> 300
	SYLLRNP	++	++	100 $\pm$ 25
	SALLRNP	—	—	—
	SFLLKNP	+++	+++	50 $\pm$ 10
	-FLLRNP	—	—	—
	S-LLRNP	—	—	—
	SF-LRNP	—	—	—
	negative-charge cluster			
(P54–L66)	PFWEKNEESGL (P-13-L)	—	—	—
Chinese hamster receptor sequence				
(42–55)	SFFLRNP G ENTFEL (ChS-14-F)	+++	+++	15 $\pm$ 5
(42–48)	SFFLRNP (ChS-7-P)	+++	+++	50 $\pm$ 10

Table 2 Stimulation of phospholipase C activity by human thrombin-receptor-activating peptides in CCL39 cells

(a) [^3H]inositol phosphate formation in CCL39 cells was determined in the presence of 20 mM LiCl as described in the Materials and methods section following 10 min of stimulation by the indicated concentration of α -thrombin or human α -thrombin-receptor peptides (indicated by single-letter amino-acid code). Fold stimulation by agonists relative to basal accumulation of [^3H]inositol phosphate, measured in the presence of LiCl alone, is presented. The range of error between duplicate determinations is indicated, as well as the percentage maximal activity obtained using 30 μM SFLLRNP. (b) Initial rates of [^3H]inositol phosphate formation were determined following 1 min of stimulation by 1 nM α -thrombin or 10 μM peptide agonists. The mean c.p.m. from duplicate measurements ($\pm$ range of error) and maximal activity (%) (stimulated by SFLLRNP), are shown. DTT (50 μM) was present in the incubation where indicated. Similar results were obtained in two or more independent experiments.

(A) [^3H]inositol phosphate formation during a 10 min incubation period

Agonist	Concn. (μM)	Stimulation (fold)	Maximal activity (%)
Thrombin	0.001	5.9 ± 0.2	83
SFLLRNP	1	3.7 ± 0.1	52
	3	5.7 ± 0.2	80
	10	6.6 ± 0.2	93
	30	7.1 ± 0.4	100
SFLLKNP	30	7.7 ± 0.1	108
MFLLRNP	30	6.4 ± 0.2	90
SYLLRNP	30	4.3 ± 0.2	61
prop FLLRNP	30	1.7 ± 0.2	24
YFLLRNP	30	1.4 ± 0.2	20

(B) Initial rates of [^3H]inositol phosphate formation by peptides (10 μM)

Agonist	[^3H]inositol phosphates (c.p.m./min)	Maximal activity (%)
Thrombin (1 nM)	690 ± 38	56
Thrombin (1 nM) + DTT	632 ± 32	53
SFLLRNP	1733 ± 63	100
SFLLRNP + DTT	1648 ± 153	96
SFLLRNP	1911 ± 47	114
TFLLRNP	1551 ± 145	93
AFLLRNP	2331 ± 113	128
CFLLRNP	765 ± 13	60
CFLLRNP + DTT	1004 ± 8	71

incubation period. Seven of the peptides had either decreased (SYLLRNP, **propionyl**FLLRNP, YFLLRNP; Table 2a) or no (-FLLRNP, S-LLRNP, SF-LRNP and SALLRNP; not shown) agonist activity. (Residues shown in bold represent residue substitutions and hyphens indicate deletions.) Peptides displaying no activity were inactive at concentrations up to 300 μM . Peptides which retained agonist activity were compared by assaying initial rates of ^3H -inositol phosphate formation as shown in Table 2(b). Under these conditions, no major decrease in activity, as compared with the wild-type human or hamster peptide sequence, was observed for peptides with N-terminal substitutions of serine with threonine, alanine, methionine (not shown) or cysteine. CFLLRNP was found to be more effective in the presence of DTT (to block disulphide formation between free cysteine residues) at a concentration of 50 μM , which did not interfere with α -thrombin-induced inositol phosphate release.

Thus, as presented in Table 2, replacement of the N-terminal serine residue with a small non-charged amino acid such as threonine, alanine, methionine or cysteine, had little or no effect on the ability of the peptides to activate phospholipase C in

CCL39 cells. Peptide agonist activity was significantly reduced, however, when the serine was either replaced with propionic acid (desaminoalanine), with tyrosine, or omitted (see below).

Concerning the amino acid in the second position, replacement of phenylalanine by tyrosine (SYLLRNP) diminished the ability of the peptide phospholipase C, whereas its replacement by alanine (SALLRNP) nearly abolished activity. As can be seen in Table 1a, the level of stimulation obtained with 30 μM SYLLRNP represents 61 % of that obtained with 30 μM SFLLRNP. We found that the positively charged arginine in the fifth position could be replaced by lysine (SFLLKNP) without affecting activity. Finally, deletions of the first, second or third most N-terminal residues of α -thrombin-receptor-activating peptides destroyed their activity (Table 1). None of the inactive peptides examined was able to antagonize the activation of phospholipase C by α -thrombin, or by SFLLRNP at up to 100-fold greater concentrations (results not shown).

In addition to the tethered-ligand peptides, we also examined the activity of a 13-residue peptide (P-13-L) corresponding to the 'negative-charge cluster' of the receptor (Table 1). This highly acidic motif resembles a sequence present on the thrombin inhibitor hirudin that mediates its interaction with the anion-binding exosite I of α -thrombin [15]. A peptide containing the same sequence has been shown to bind to α -thrombin and modify activity of the protease [16], however, P-13-L displayed no agonist or antagonist activity in the phospholipase C assay (Table 1 and data not shown).

Adenylyl cyclase inhibition

In intact CCL39 hamster fibroblasts, α -thrombin and synthetic receptor peptide agonists activate a G_i -coupled intracellular signalling pathway which can be detected by inhibition of forskolin-stimulated adenylyl cyclase. Seven-residue peptides derived from both Chinese hamster [6] and human receptor sequence (results not shown) inhibit forskolin-stimulated adenylyl cyclase activity of cells by approx. 40 %, with a half-maximal effect observed at approx. 3 μM . Structure-activity analyses of adenylyl cyclase inhibition revealed that the ability of modified human receptor peptides to inhibit adenylyl cyclase paralleled their ability to activate phospholipase C (Table 1).

DNA synthesis re-initiation

Although α -thrombin is a potent mitogen for CCL39 cells, α -thrombin-receptor-activating peptides fail to elicit a mitogenic response. However, stimulation of DNA synthesis by thrombin-receptor peptides in CCL39 cells can be revealed by the presence of tyrosine kinase-activating growth factors such as FGF [6]. Therefore, the effect of receptor peptides on cell cycle re-entry in quiescent CCL39 cells was evaluated both in the absence and presence of FGF. As presented in Figure 1(a), N-terminal amino-acid substitutions that did not affect the ability of the peptides to activate G-protein-coupled effectors had no effect on the ability of peptides to cooperate with FGF for stimulation of DNA synthesis in CCL39 cells. Correspondingly, sequence alterations that severely diminished or abolished the ability of receptor-activating peptides to activate phospholipase C, including YFLLRNP, SALLRNP and deletions, also inhibited their ability to potentiate [m - ^3H]thymidine incorporation in CCL39 cells. Modified peptides devoid of activity had no antagonist effect on α -thrombin-stimulated DNA synthesis (Figure 1b).

To define further the dose-dependency of the mitogenic signalling of α -thrombin-receptor peptides, we used a tumor-

Figure 1 Stimulation of DNA synthesis re-initiation in quiescent CCL39 cells by α -thrombin and receptor-activating peptides

(a) $[m\text{-}^3\text{H}]\text{thymidine}$ incorporation was determined after 24 h in the presence of synthetic peptide in the absence (open bars) or presence (solid bars) of 25 ng/ml FGF. A 5-fold molar excess of DTT was included in the incubation where indicated. (b) α -Thrombin-stimulated $[m\text{-}^3\text{H}]\text{thymidine}$ incorporation was measured alone (solid bars) or in the presence of 100 μM of the indicated peptide. Results are presented as percentages of maximal stimulation obtained with 10% (v/v) FCS with error being $\leq 2\%$.

igenic variant of the CCL39 line, A71 cells. A71 cells display a strikingly increased sensitivity to the mitogenic effects of α -thrombin (EC_{50} approx 4×10^{-12} M, as opposed to approx. 3×10^{-9} M in CCL39 cells) [11], and receptor-activating peptides are potent inducers of DNA synthesis re-initiation. In the absence of added growth factors, α -thrombin-receptor-activating peptides stimulate approx. 75% of the maximal $[m\text{-}^3\text{H}]\text{thymidine}$ incorporation obtained with 10% (v/v) FCS. Therefore, using stimulation of DNA synthesis in A71 cells as a read-out system, we determined EC_{50} values for each of the active peptides (see Table 1). It is noteworthy that the dose-dependency of this effect in A71 cells by receptor peptides is similar to that seen for CCL39 cells in the presence of FGF (results not shown). The 14-residue hamster or human receptor-activating peptides were found to be 3–5 times more potent than their shorter counterparts at stimulating synthesis of DNA in A71 cells (Table 1). We have observed previously a similar increased potency of 14-residue peptide compared with 7-residue hamster receptor peptide for DNA synthesis stimulation in CCL39 cells, whereas inversely the shorter peptide was more effective than the longer one at

stimulating phospholipase C activation and adenylyl cyclase inhibition [6].

DISCUSSION

The observed effects *in vitro* of α -thrombin on platelets, inflammatory cells, the vascular endothelium, underlying smooth muscle cells and connective tissue point to an important role for α -thrombin in normal haemostasis and vessel repair processes, as well as a possible involvement of the enzyme in thrombotic, inflammatory and proliferative processes associated with disease states (recently reviewed in [17]). Indeed, results from animal studies using specific thrombin inhibitors support a role for α -thrombin in different models of platelet thrombosis [18–21] and in the restenosis process after balloon angioplasty [22]. Thus, the α -thrombin receptor appears to be a promising target for the development of therapeutics with anti-thrombotic or anti-proliferative properties. Understanding of α -thrombin-receptor activation at a molecular level would provide the basis for design of pharmacological agents that block, or selectively activate receptors on defined target cells.

The studies described above allow us to draw certain conclusions concerning the structural features of a peptide ligand required for α -thrombin-receptor activation. First, we observed that the N-terminal serine could be replaced by several other amino acids, including alanine, threonine, cysteine or methionine without significant loss of activity. Thus the hydroxyl group on the serine residue is apparently not involved in receptor interaction. These observations are in agreement with recent findings by Vassallo et al. [5] indicating that receptor hexapeptides with alanine or threonine in the N-terminal position only slightly affect their ability to cause human platelet aggregation. Removal of the NH_2 group (propionyl FLLRNP), however, significantly reduced peptide activity, in accordance with two recent reports indicating that the N-terminal amino group plays an essential role in receptor activation [23, 24]. We also found that replacing the serine by tyrosine (aromatic side-chain) significantly reduced agonist activity.

Thus, for the N-terminal residue the following salient features can be noted: (1) the N-terminal serine can be replaced by small or intermediate-sized amino acids ($\pm$ hydroxyl groups) except those possessing aromatic side-chains (phenylalanine and tyrosine); and (2) the primary amino group is essential for activity (ionic interaction). The second residue must be aromatic (phenylalanine or tyrosine) to preserve agonist activity. Deletion of residues in any of the first three positions abolishes activity, emphasizing the constraint of spacing, probably between the N-terminal amino group, the aromatic ring in the second position and some additional moiety positioned after the third amino acid. This additional group may be the fifth-position arginine side-chain and/or possibly the fourth-position leucine side-chain, since shortening SFLLR to SFLL or SFL [6,25] or replacing the fourth or fifth residues with alanine (SFLLA or SFLLAR) [5,23,24] dramatically decreases agonist activity. However, the conservative exchange of the fifth-position arginine with lysine did not change the peptide's ability to activate phospholipase C (shown here), and its replacement by alanine in a mutant receptor expressed in *Xenopus* oocytes did not change the response to saturating α -thrombin concentrations [23]. Thus, the role of the fifth-position arginine may not be to form a strictly defined ionic interaction with the receptor-binding site, but rather to keep the peptide in a favourable conformation. If so, the only potential point of ionic interaction between 'ligand' and receptor would be in the N-terminal amino group, while the rest would be hydrophobic interaction.

Two distinct G-protein signalling pathways stimulated by α -thrombin and receptor peptide agonists have been well characterized in CCL39 hamster fibroblasts. One is mediated by a 'Gq-like' protein coupled to phospholipase C activation and a second, by a pertussis toxin-sensitive G_i protein that inhibits forskolin-stimulated adenylyl cyclase activity (for review see [13]). The parallel activation pattern for each of these events by modified peptides would suggest that the same α -thrombin/peptide receptor is able to interact with the α -subunits of both of these G proteins. We cannot exclude the possibility, however, that different structurally similar receptor subtypes co-exist on hamster fibroblasts. Nevertheless, the similar structure–activity relationship observed for activation of hamster fibroblasts (this study) and human platelets [5] by modified peptides indicates that the human platelet and hamster fibroblast receptors, which share only 79% overall amino-acid-sequence similarity [2], have common structure–function properties.

Concerning the site of peptide interaction with the receptor, current models for the secondary and tertiary structure of G-protein-coupled receptors largely based on analogy to bacteriorhodopsin [26,27], and recently determined by hydrophobic-cluster analysis [28], would suggest that the seven α -helices of these receptors may form a ligand-binding pocket. Studies on adrenergic, muscarinic and dopaminergic receptors (reviewed in [29]) would support this model, although data on the ligand-binding sites of peptide receptors is not yet available. Strategies involving domain-swapping between peptide receptors with different ligand specificity and *in vitro* mutagenesis should provide useful insights into this question.

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