Destabilization of AT₁ Receptor mRNA by Calreticulin

Georg Nickenig,* Frank Michaelsen,* Cornelius Müller, Anja Berger, Thomas Vogel, Agapios Sachinidis, Hans Vetter, Michael Böhm

Abstract—AT₁ receptor activation leads to vasoconstriction, blood pressure increase, free radical release, and cell growth. AT₁ receptor regulation contributes to the adaptation of the renin-angiotensin system to long-term stimulation and serves as explanation for the involvement of the AT₁ receptor in the pathogenesis of cardiovascular disease. The molecular mechanisms involved in AT₁ receptor regulation are poorly understood. Here, we report that angiotensin II accelerates AT₁ receptor mRNA decay in vascular smooth muscle cells. A cognate mRNA region within the 3’ untranslated region at bases 2175 to 2195 governs the inducible decay of the AT₁ receptor mRNA. Sequential protein purifications led to the discovery of a novel mRNA binding protein, calreticulin, which mediates destabilization of the AT₁ receptor mRNA. Angiotensin II–caused phosphorylation of calreticulin enables binding of calreticulin to the AT₁ receptor mRNA at bases 2175 to 2195 and propagates calreticulin-induced acceleration of AT₁ receptor mRNA decay. Thus, a novel mRNA binding protein, calreticulin, is discovered, which causes AT₁ receptor mRNA degradation via binding to a distinct mRNA region in the 3’ untranslated region. These findings display a novel mechanism of posttranscriptional mRNA processing. (Circ Res. 2002;90:53-58.)

Key Words: angiotensin II ■ AT₁ receptor ■ mRNA binding protein ■ mRNA stability ■ calreticulin

The renin-angiotensin system (RAS) is central for the physiological regulation of blood pressure and fluid homeostasis. Importantly, the RAS has been strongly implemented in the pathogenesis of hypertension and atherosclerosis. This assumption is predominantly based on numerous interventional studies demonstrating that pharmacological blockade of the RAS leads to diminished vascular damage, to an improved endothelial function, and ultimately to reduced mortality rates in patients suffering from hypertension, coronary heart disease, or heart failure. Most biological effects of the RAS such as vasoconstriction, neurohumoral activation, cell growth, and free radical release are mediated through the AT₁ receptor. The expression of the AT₁ receptor is altered by angiotensin II, growth factors, estrogen, and lipoproteins. Dysregulated expression of AT₁ receptors may profoundly participate in the development of vascular damage. AT₁ receptor regulation takes place at the posttranscriptional level via agonist-induced (de)stabilization of the AT₁ receptor mRNA. As previously shown, a family of mRNA binding proteins binds the AT₁ receptor mRNA at bases 2175 to 2195 and propagates AT₁ receptor mRNA decay.

Materials and Methods

Cell Culture

Vascular smooth muscle cells (VSMCs) were isolated from rat thoracic aorta by enzymatic dispersion and cultured over several passages. Experiments were performed with cells from passage 5 to 15.

mRNA Isolation, Northern Analysis, and Polymerase Chain Reaction

Cells were lysed with 1 mL RNA-clean (AGS), scraped, and processed according to the manufacturer’s protocol in order to obtain total cellular RNA. Northern blots were prehybridized for 2 hours at 42°C and then hybridized for 15 hours at 42°C with a random-primed, [³²P]-dCTP–labeled, rat AT₁ receptor cDNA probe. The rat AT₁ receptor cDNA probe was a 479-bp fragment generated from an AT₁ receptor cDNA template by the polymerase chain reaction (PCR) using the same primer pair as mentioned in the PCR section. Isolated total RNA (2 µg) and the mutAT₁ mRNA (10 pg) were mixed and reverse transcribed using random primers. The single-stranded cDNA was amplified by PCR using Taq DNA-polymerase (Boehringer). Twenty eight cycles were performed under the following conditions: 30 seconds, 94°C; 55°C, 45 seconds; and 72°C, 45 seconds. The sequence for AT₁ receptor sense and antisense primers were 5’-ACCCCTCTACAGCATCATCTTTGTGGTGGA-3’ and 5’-GGCGGCGTCCGATTTCCCGAGACTCTATAATGAGA-3’, respectively. The same samples were used for GAPDH cDNA amplification to confirm that equal amounts of RNA were reverse transcribed. The primers employed were 5’-ACCACAGTTCCATCATC-3’ and 5’-TCCACACCTGGTGTGTA-3’. PCR amplification gave 478...
bp and 452 bp of fragments originated from the AT₁ receptor mRNA and GAPDH mRNA, respectively.

**UV mRNA Protein Crosslink Assays**

Polysomal protein (10 µg) was mixed on ice with 4 to 10 pmol of [³²P] UTP-labeled RNA transcripts (4×10⁸–10⁹ cpm) and UV mRNA protein crosslink experiments were performed as described earlier.¹²

**Cell Transfections and Calreticulin Constructs**

For electroporation, VSMCs grown at a confluent monolayer were removed from the culture dish by addition of trypsin and pelleted. The pellet was resuspended in 200 µL of OptiMEM I (Gibco BRL Life Technologies). Samples (10⁶ cells) were incubated with 20 µg of the respective DNA (pcDNA3 vector, calreticulin full length cDNA) in precooled cuvettes (Promega) for 30 minutes on ice. Electroporation was performed for 16 ms at 0.3 kV and 500 µF. Cells were plated on the appropriate culture dishes or microtiter plates. Full-length calreticulin was subcloned into the eucaryotic expression vector pcDNA3 (Invitrogen). A calreticulin antisense oligonucleotide 5'-AGGTGTCAGACCGAAGGAGCAG-3' (antisense) and the Abi Prism 7700 Sequence Detector. PCR instructions using the Sybr-Green Mastermix Kit (Applied Biosystems) and the HindHI restriction sites.

**Anionic Exchange Chromatography**

Polysomal protein extracts were used for an initial purification step on a standard chromatography system using a Source DEAE Sepharose Fast Flow column (Pharmacia Biotech) equilibrated in a buffer containing 10 mmol/L Tris-HCl (pH 7.4) and 0.5 mmol/L EDTA. Proteins were eluted in a NaCl-gradient from 100 mmol/L up to 1 mol/L. After desalting, protein fractions with RNA-binding activity were submitted to a second anionic exchange chromatography using a FPLC chromatography system with a Q-Sepharose HP column (Pharmacia Biotech). Elution gradient was between 500 and 1 mol/L NaCl. Binding proteins were excised out of SDS-PAGE gels and identified via MALDI mass spectroscopy.

**Immunoprecipitation and Western Blotting**

Stimulated VSMCs were washed and lysed. After centrifugation, supernatants were incubated with an anti-calreticulin antibody (Novus Biologicals). Protein-A Sepharose (20 mg) was added and incubated for 1 hour. After centrifugation, pellets were washed and samples were boiled, electrophoresed, and blotted to nitrocellulose membranes. Immunoblotting/detection was performed with an anti-phosphotyrosine antibody, clone 4G10 (Upstate Biotechnology), an anti-mouse peroxidase conjugate antibody (Sigma, Taufkirchen, Germany), and the enhanced chemiluminescence (ECL) kit (Pharmacia Biotech).

**In Vitro Phosphorylation of Calreticulin**

In vitro phosphorylation of calreticulin was performed according to the manufacturer’s protocol using the following: β-insulin receptor kinase/Src kinase/JNK kinase kit (all obtained from Stratagene).

**Real-Time PCR**

For quantitative PCR, a reverse transcription was performed (primer for AT₁ receptor 5′-GAGGTAAACATACATTGCC-3′, GAPDH 5′-TGTATGCTGGGATTTGGA-3′). After 1:1000 dilution, the PCR was performed according to the manufacturer’s instructions using the Sybr-Green Mastermix Kit (Applied Biosystems) and the ABI prism 7700 Sequence Detector. PCR primer: AT₁ receptor 5′-GAGGTAAACATACATTGCC-3′ (sense) and 5′-GAGGTAAACATACATTGCC-3′ (antisense); GAPDH 5′-TGTATGCTGGGATTTGGA-3′ (sense) and TGATACCCAGCTGGAGAGGAGCAC-3′ (antisense).

**Statistical Analysis**

Data are presented as mean±standard error of mean (SEM) obtained in at least 3 separate experiments. Statistical analysis was performed using the ANOVA test. A value of P<0.05 indicates statistical significance.

**AT₁ Receptor Decay**

It is well established that angiotensin II downregulates AT₁ receptor mRNA expression. The putative angiotensin II–elicited acceleration of AT₁ receptor mRNA was tested in an in vitro decay assay. VSMCs were incubated with vehicle or 100 nmol/L angiotensin II for 2 hours, polyribosomes were isolated, and the decay of the AT₁ receptor mRNA was monitored in an in vitro system. Figure 1 shows that angiotensin II causes an accelerated AT₁ receptor mRNA decay within the polysomal compartment. Thus, angiotensin II causes destabilization of AT₁ receptor mRNA in VSMCs.

**Binding of Polysomal Proteins to the AT₁ Receptor mRNA**

Polysomal proteins and their binding to the 3′ untranslated region are involved in the induced AT₁ receptor mRNA destabilization. UV mRNA protein crosslink assays were used to analyze protein-mRNA interactions in the AT₁ receptor mRNA. Polysomal proteins isolated from VSMCs were incubated with a radioactive AT₁ receptor mRNA riboprobe of the region 1864 to 2213 in the presence of unlabeled, mutated AT₁ receptor mRNA transcripts. Figure 2A shows a representative autoradiography, which reveals that several polysomal proteins bind the 3′ untranslated region of the AT₁ receptor mRNA. This binding is selective and is inhibited by addition of unlabeled AT₁ receptor mRNA transcripts bases 1864 to 2213 and 2131 to 2213 but not by fragment bases 2131 to 2187 and 2131 to 2170, suggesting binding of the identified proteins at the very 3′ part of the AT₁ receptor mRNA. Previous studies with mutated competitors showed that the cognate binding sequence is located at bases 2175 to 2195. Control experiments using multiple AT₁ receptor mRNA competitors and various radiolabeled AT₁ receptor mRNA probes (5′ untranslated region, open reading frame)

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**Figure 1.** Angiotensin II destabilizes AT₁ receptor mRNA in VSMCs. VSMCs were incubated for 2 hours with vehicle or 100 nmol/L angiotensin before polysomes were isolated, which were incubated with an energy-generating system for the indicated time points, RNA was isolated, and AT₁ receptor mRNA was quantified by Northern blots. Densitometric analysis. 18S and GAPDH RNA expression remained stable over the experimental period (n=5, mean±SEM; *P<0.05).
demonstrated that mRNA binding proteins exclusively bind within the delineated region bases 2175 to 2195. This region comprises a AUUUUA hexamer and is considerably AU-rich (5’-AAAGUAUUUUAUGUAUGU-3’).

**Isolation and Characterization of Calreticulin as AT 1 Receptor mRNA Binding Protein**

Further clarification of the described regulative pathways is dependent on the identification of participating binding proteins. Polysomal proteins were isolated from VSMCs and subjected to sequential anionic exchange chromatography. Isolated fractions were monitored by UV crosslink assays (Figure 3A). The left panel of Figure 3A shows the initial purification step on a standard chromatography system using a Source DEAE-Sepharose Fast Flow column. Protein fractions with RNA-binding activity (lanes 7 and 8) were submitted to a second anionic exchange chromatography using a FPLC chromatography system (right panel). A purified protein (right panel, lanes 8 and 9) was extracted. Protein sequence was defined by MALDI analysis and revealed identification of calreticulin. Figure 3B shows amino acid sequences of fragments characterized during fingerprinting. Homology alignments identified calreticulin as the purified protein.

Control experiments showed that recombinant calreticulin binds indeed to the 3’ untranslated region of the AT 1 receptor mRNA. However, this requires phosphorylation of calreticulin. Recombinant calreticulin was phosphorylated with Src kinase, JNK kinase, β-insulin receptor kinase (β-IR kinase), or autophosphorylated. Binding of calreticulin to the AT 1 receptor riboprobe bases 1864 to 2213 was monitored by UV crosslink assay. Figure 3C reveals that phosphorylation with Src as well as JNK kinase enables interaction of calreticulin with the AT 1 receptor mRNA.

In order to assess whether this different binding of calreticulin was based on differential protein phosphorylation, the latter was monitored. Figure 3D shows that JNK kinase led to a profound phosphorylation of calreticulin. Src kinase also reproducibly phosphorylated calreticulin, but not as abundant as JNK. β-IR kinase and autophosphorylation, which did not result in binding of calreticulin to the AT 1 receptor mRNA, showed no detectable phosphorylation of calreticulin. Thus, phosphorylation of calreticulin is required for interaction with the AT 1 receptor mRNA; however, the extent of phosphorylation seems not to influence the strength of protein-mRNA cross-talk.

Calreticulin binds the AT 1 receptor mRNA in the region 2175 to 2195, as confirmed by UV crosslink assays including various competitors (Figure 3E). Calreticulin did not bind to the GAPDH mRNA, eNOS mRNA, or to an AT 1 receptor mRNA lacking bases 2175 to 2195 (data not shown).

**Phosphorylation of Calreticulin by Angiotensin II**

As angiotensin II accelerates AT 1 receptor mRNA decay and as calreticulin-AT 1 receptor mRNA interaction requires phosphorylation, the ability of angiotensin II to phosphorylate calreticulin was investigated. VSMCs were stimulated with 100 nM angiotensin II, calreticulin was immunoprecipitated from cell homogenates and phosphorylation was monitored with an anti-phosphotyrosine–antibody and Western blotting (Figure 4), revealing that angiotensin II led to a time-dependent phosphorylation of calreticulin.

These experimental findings identify calreticulin as an mRNA binding protein that interacts with the 3’ untranslated region of the AT 1 receptor mRNA at bases 2175 to 2195, provided that phosphorylation was initiated. The latter is inducible on stimulation with angiotensin II.

**Calreticulin Accelerates AT 1 Receptor mRNA Decay**

The functional relevance of the interaction of calreticulin with the AT 1 receptor mRNA was assessed in transfection experiments. Overexpression of calreticulin in VSMCs downregulated basal AT 1 receptor expression (Figure 5A).

To assess whether the AT 1 receptor mRNA is destabilized by calreticulin, recombinant, phosphorylated calreticulin was added to in vitro decay assays. AT 1 receptor mRNA concentrations were assessed by real-time PCR. Figure 5B demonstrates that calreticulin led to a significant destabilization of the AT 1 receptor mRNA.

In addition, it was tested whether angiotensin II–induced AT 1 receptor mRNA downregulation, which is based on AT 1 receptor mRNA destabilization, is inhibited by transfection with antisense-calreticulin cDNA. Therefore, cells were transfected with either an insertless pcDNA3 vector or an antisense calreticulin construct and incubated with vehicle or 1 μM/L angiotensin II for 4 hours. Effective overexpression of calreticulin or inhibition of calreticulin protein expression

**Figure 2.** Polysomal proteins bind the 3’ untranslated region of the AT 1 receptor mRNA. In vitro transcribed, 32P-UTP-labeled AT 1 receptor mRNA (bases 1864 to 2213) was incubated with polysomal proteins isolated from VSMCs in culture. RNA and protein were crosslinked via UV irradiation and unbound RNA was digested with RNases. The reaction was electrophoresed through SDS-PAGE and radioactively labeled proteins were visualized by autoradiography. Representative autoradiogram of polysomal proteins isolated from VSMCs in culture. RNA and some reaction included 30- and 5-fold excess of unlabeled AT 1 receptor mRNA bases 1864 to 2213 crosslinked to polysomal proteins isolated from VSMCs. As indicated on the top, some reaction included 30- and 5-fold excess of unlabeled AT 1 receptor mRNA competitors bases 1864 to 2213, 2131 to 2213, 2131 to 2187, and 2131 to 2170. Lane 10 displays a negative control in which no protein was added. The riboprobe was completely digested. Lane 11 displays a positive control of the riboprobe in which RNase digestion was omitted.
was monitored by Western blots (insert at the top of Figure 5C). AT1 receptor and GAPDH mRNA were quantified by real-time PCR. Figure 5C demonstrates that antisense calreticulin effectively blocks the angiotensin II–caused AT1 receptor mRNA decrease, suggesting that interaction of calreticulin with the 3′/H11032 untranslated region of the AT1 receptor mRNA causes destabilization of the AT1 receptor mRNA.

Discussion
The presented data display a cognate mRNA sequence involved in posttranscriptional regulation of the AT1 receptor, and the identification of a novel mRNA binding protein is reported. Calreticulin binds, if phosphorylated, to the cognate sequence bases 2175 to 2195 of the AT1 receptor mRNA and leads to destabilization of the AT1 receptor mRNA. Angiotensin II stimulation, which causes destabilization of AT1 receptor mRNA, causes phosphorylation of calreticulin.

Cognate sequences within the 3′ untranslated region, such as the pentamer AUUUA, and nucleotide sequences, such as UUAUUUA(U/A)(U/A) and UUAUUUAUU, regulate mRNA stability by interaction with cytosolic and nuclear-associated factors.13–20 Several genes have been explored with regard to mRNA binding protein properties and the role of the mRNA consensus sequences in the homeostasis of mRNA turnover, including immediate early genes (eg, c-fos, c-myc), cytokines (eg, colony stimulating factor, tumor necrosis factor α), growth factors (eg, vascular endothelial growth factor), and the inducible and endothelial isoforms of nitric oxide synthase.21–29 Regulation of endothelial NO synthase expression is connected to a 51-kDa binding protein interacting with a 43 base sequence in the 3′ untranslated region of the eNOS mRNA.30 Malbon and colleagues have

Figure 3. Identification of the AT1 receptor mRNA binding protein calreticulin. A, Polysomal proteins were isolated and subjected to sequential anionic exchange chromatographies. Eluted fractions were monitored with crosslink assays (riboprobe AT1 receptor mRNA 1864 to 2213). Proteins on lanes 8 and 9 (right gel) were separated by electrophoresis, the corresponding protein was excised, eluted, and characterized by MALDI. N indicates no protein; P, probe without digestion. B, Amino acid sequences of protein fragments derived from fingerprinting analysis following MALDI. Alignment with known protein sequences using Profound searches revealed homology with calreticulin. C, Phosphorylated calreticulin binds to the AT1 receptor mRNA. Recombinant calreticulin (cal) was phosphorylated with Src kinase, JNK kinase, β-insulin receptor kinase (β-IR kinase), or autophosphorylated. Binding of calreticulin to the AT1 receptor riboprobe bases 1864 to 2213 was monitored by UV crosslink assay. When UV crosslinking of calreticulin to the AT1 receptor mRNA is omitted, no binding activity is visualized (negative control). D, Calreticulin was phosphorylated with Src kinase, JNK kinase, β-IR kinase, or autophosphorylated in the presence of 32P-ATP. Reactions were separated on a acrylamide gel. Phosphorylated calreticulin was detected by autoradiography. E, Phosphorylated calreticulin was incubated and crosslinked with the AT1 receptor riboprobe bases 1864 to 2213 in the presence of the AT1 receptor mRNA competitors bases 1864 to 2213, 2175 to 2195, and the unspecific mRNA competitor GAPDH. The binding pattern reveals that calreticulin binds the AT1 receptor mRNA at bases 2175 to 2195.
thoroughly characterized interactions of the β2-adrenergic receptor mRNA with corresponding binding proteins.17,25,26 Their work revealed that mRNA processing is arranged through binding of various proteins in the 3' untranslated region of the β2-adrenergic receptor mRNA, including a 35-kDa protein. The latter is induced on stimulation with β-adrenergic agonists and is a prerequisite for agonist-induced destabilization of the β2-adrenergic receptor mRNA acting through cognate sequences identified in the 3’ untranslated region composed of a 20 nucleotide (A+U)-rich element that compromises an AUUUUA hexamer rather than the commonly identified AUUUA pentamer.17 The β2-adrenergic receptor mRNA displays also binding to AUU-1, which probably resembles the best known mRNA binding protein.31 This 35 kDa binding protein that was cloned and functionally characterized by Brewer participates also in β2-adrenergic receptor mRNA and seems to be involved in the posttranscriptional processing of various other genes.14

Several other mRNA binding proteins ranging in size from approximately 100 to 20 kDa have been described, although only a few of them have been characterized on the level of nucleotide or amino acid sequence.14,15,27,32

The characterization of the novel mRNA binding protein calreticulin further elucidates the detailed molecular pathways of AT1 receptor mRNA turnover. So far, calreticulin has been ascribed 3 different functions. First, calreticulin is involved in intracellular calcium homeostasis due to calcium binding sites in the protein and its ability to regulate other calcium-handling proteins.33 Second, calreticulin acts as chaperone-like molecule involved in folding and oligomerization of glycoproteins.34 Third, calreticulin has been described as a receptor for nuclear export.35 The data presented herein associate calreticulin with another biological property: based on its binding to distinct nucleotides in the 3’ untranslated region of the AT1 receptor mRNA, calreticulin is engaged in the inducible decay of the AT1 receptor mRNA. This result is in concert with the finding that calreticulin acts as mRNA binding protein in rubella virus.36 Obviously, phosphorylation causes a either a conformational change of calreticulin or activates a preformed binding site of calreticulin, which enables the interaction with the mRNA. It cannot be excluded that calreticulin binds other protein factors before or while interacting with the target mRNA. Furthermore, it is not clear if calreticulin itself activates RNases, which realize the actual AT1 receptor mRNA decay. Alternatively, calreticulin may induce a change in the tertiary structure of an mRNA, leading to a more or less pronounced interference with nucleases.

Binding of mRNA binding proteins on their corresponding mRNA is profoundly influenced by secondary and tertiary structures of the RNA. Hairpins or stem loops formed by the RNA region of interest may interact with neighboring proteins. In the case of the AT1 receptor, computer modeling showed that the identified AT1 receptor mRNA binding motif bases 2175 to 2195 forms such a stem loop. That holds true for the entire AT1 receptor mRNA and also for the isolated 20-base transcript used in our study as competitor and decoy. In concert with our finding that such a mutated mRNA binds no longer to polysomal proteins, deletion of this motif

Figure 5. Calreticulin destabilizes the AT1 receptor mRNA. A, Full-length calreticulin cDNA cloned in the expression vector pcDNA3 (cal) or the insert-less pcDNA3 (control) were transfected in VSMCs via electroporation technique. Overexpression and transfection efficiency above 20% was assured. After 48 hours, AT1 receptor mRNA expression was monitored with Northern blotting. Calreticulin overexpression led to a significant downregulation of AT1 receptor mRNA. 18S RNA remained unaltered. Densitometric analysis of 5 separate experiments. B, Either recombinant phosphorylated calreticulin (AT1-Cal) or the kinase reaction without calreticulin (AT1-control) were added to an in vitro decay assay including the in vitro transcribed AT1 receptor mRNA. Degradation of AT1 receptor mRNA was assessed by real-time PCR. As internal control, a GAPDH mRNA was included in the reaction and concomitantly quantified (GAPDH-Cal) (n=3, mean±SEM; *P<0.05). C, Cells were transfected with either an insert-less pcDNA3 vector (control and angII) or an antisense calreticulin construct (AS-Cal). The effect of antisense calreticulin and sense calreticulin (S-Cal). Construct transfection was monitored by Western blots (insert). Twenty-four hours later VSMCs were incubated with vehicle (control) or 1 μmol/L angiotensin II (angII) for 4 hours. AT1 receptor and GAPDH mRNA were quantified by real-time PCR (n=5, mean±SEM; *P<0.05).
abolished the stem loop; therefore, suggesting the importance of secondary structure for protein-mRNA interaction.

The presented findings could represent general mechanisms involved in mRNA processing and could be also applicable to other genes. However, calreticulin binds neither to the GAPDH nor to the eNOS mRNA.

AT1 receptor regulation is of broad interest because numerous studies have shown that AT1 receptor activation is closely involved in the pathogenesis of hypertension, atherosclerosis, and heart failure.1–6 The identification of the involved binding protein and the cognate mRNA sequence enables investigations that need to test if dysregulated AT1 receptor expression induced by inherited variations in calreticulin or the corresponding 3′ untranslated AT1 receptor mRNA is involved in the development of cardiovascular diseases.

Our findings reveal novel molecular mechanisms involved in posttranscriptional regulation of the AT1 receptor mRNA, prompting further characterization of interactions between binding proteins and the AT1 receptor mRNA, and thus, enabling the structural identification of engaged binding factors. The latter is a prerequisite for the better understanding of the complex cellular mechanisms of cytosolic mRNA turnover. In addition, the described mechanisms for AT1 receptor regulation may have relevant implications for the pathogenesis of atherosclerosis and hypertension because pathological abnormalities of AT1 receptor regulation may drive both development and progression of these diseases.

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