Development of a Genotype 325–Specific proCPU/TAFI ELISA

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Objective—A Thr/Ile polymorphism at position 325 in the coding region of proCPU has been reported. Immunological assays, fully characterized (including genotype dependency), are required for the quantitation of proCPU levels.

Methods and Results—We have generated a panel of monoclonal antibodies against human, plasma-derived proCPU. Two combinations exhibiting distinct reactivities were selected for measurement of proCPU in plasma. T12D11/T28G6-HRP yielded values of 10.1±3.1 µg/mL (mean±SD, n=86; normal donors), and T32F6/T9G12-HRP yielded values of 5.4±3.0 µg/mL. Grouping according to the 325 genotype demonstrated that T12D11/T28G6-HRP was independent to this polymorphism whereas T32F6/T9G12-HRP revealed a complete lack of reactivity with the Ile/Ile genotype (ie, 0±0.0, 4.2±1.7, and 7.3±2.9 µg/mL for the Ile/Ile, Ile/Thr, and Thr/Thr isoforms, respectively). Commercially available antigen assays appeared to be partially dependent on the 325 genotype (eg, 44±8.9% and 100±30% for the Ile/Ile and Thr/Thr isoforms, respectively).

Conclusions—Our data demonstrate that great care should be taken when evaluating proCPU antigen values as a putative causative agent or as a diagnostic risk marker for cardiovascular events. (Arterioscler Thromb Vasc Biol. 2003;23:1122-1127.)

Key Words: TAFI  ■  proCPU  ■  monoclonal antibody  ■  ELISA  ■  genotype dependent
cide 1040 (ie, C to T substitution resulting in a Thr-to-Ile substitution at position 325). This 325 residue is of particular interest because it has been shown that the proCPU-Ile325 variant has an extended functional half-life, resulting in a 60% greater antifibrinolytic effect. Using an electroimmunoassay based on a polyclonal rabbit anti-proCPU-IgG, Brouwers et al also demonstrated that the 505A/G SNP and the proCPU-Thr147 variant is associated with apparently higher antigen levels. Another study, evaluating this 325 polymorphism to the risk of myocardial infarction using a large European multicentric case-control study, confirmed that Thr325 is associated with apparently higher antigen levels. Thus, this study suggested that Thr325 is associated with apparently higher antigen levels of a less stable TAFI variant. Brouwers et al also demonstrated that the 505A/G SNP and the 1040C/T SNP are in strong linkage disequilibrium and confirmed the findings of Henry et al\(^1\) that the proCPU-Thr147 variant is associated with apparently higher antigen levels. Another study, evaluating this 325 polymorphism to the risk of myocardial infarction using a large European multicentric case-control study, confirmed that Thr325 allele carriers have apparently higher antigen levels but that the allele frequency for this Thr325 polymorphism is not different between cases and controls, suggesting that the Thr325Ile polymorphism does not influence the risk of myocardial infarction.\(^2\)

In this study, we have generated monoclonal antibodies (MA) against human, plasma-derived proCPU. Twenty-seven monoclonals were additionally evaluated for reactivity toward various proCPU fragments. Based on these differential reactivities, 144 MA combinations were tested in sandwich-type ELISAs for the detection of proCPU and thrombin/thrombomodulin-generated fragments thereof. Subsequently, 2 combinations exhibiting distinct reactivities were selected for measurement of proCPU in plasma obtained from different proCPU genotypes. Using these assays, we demonstrate that previously suggested associations between polymorphisms in the coding region and antigen levels do not reflect differences in expression levels but rather differences in assay sensitivity between the isoforms. Importantly, such differences compromise the interpretation of epidemiological studies evaluating proCPU antigen levels as a putative risk marker for cardiovascular events.

**Methods**

**Materials**

ProCPU was purified from pooled human plasma. To generate proteolytic fragments of (pro)CPU, proCPU (3.6 \(\mu\)mol/L) was incubated with thrombin (20 mmol/L), thrombomodulin (5 mmol/L), and CaCl\(_2\) (5 mmol/L) in buffer (50 mmol/L Tris, 150 mmol/L NaCl, pH 7.4) at 37°C for 60 minutes. The reaction was stopped by addition of PPACK (30 \(\mu\)mol/L). The study population consisted of patients and controls, all participants in the HIFMECH study,\(^2\),\(^3\) a European multicenter case-control study that has as its aim the identification of differences in risk markers for myocardial infarction. Eighty-six cases and 86 controls from one center (Marseille, France) provided blood samples collected in citrate, which were centrifuged and frozen at −80°C until analysis and genotyping.

The cDNA of proCPU cloned into pBluescript SK\(^+\) was a kind gift from Dr M. Nesheim (Department of Biochemistry, Queen’s University, Kingston, Ontario, Canada). Lipofectamine 2000 and OptiMEM 1 medium containing glutamax was purchased from Invitrogen, Horseradish peroxidase (HRP)-conjugated Goat anti-mouse antibody was purchased from BioRad.

**Cell Hybridization and Screening of Hybridomas**

Monoclonal antibodies (MA) against proCPU were produced essentially as described by Galfre and Milstein.\(^4\) Briefly, Balb/c mice were immunized by subcutaneous injection of 10 \(\mu\)g proCPU (purified from plasma) in incomplete Freund’s adjuvant, followed 2 weeks later by intraperitoneal injection of 10 \(\mu\)g proCPU in incomplete Freund’s adjuvant. After an interval of at least 6 weeks, the mice were boosted intraperitoneally with 10 \(\mu\)g proCPU in saline on days 4 and 2 before the cell fusion. Spleen cells were isolated and fused with Sp2/0-Ag14 myeloma cells. After selection in hypoxanthine, aminopterin, thymidine medium, positive clones were selected by ELISA using microtiter plates coated with proCPU as capture, application of culture supernatant, and HRP-conjugated goat anti-mouse IgG for tagging. Positive clones were then used for production of ascitic fluid in pristane-primed mice.\(^5\) The IgG fraction of the monoclonal antibodies was purified from ascites by affinity chromatography on protein A sepharose.\(^6\) HRP-conjugated monoclonal antibodies were produced as described by Nakane and Kawai.\(^7\) This resulted in the selection of the following monoclonal antibodies: MA-T12D11, MA-T28G6, MA-T32F6, and MA-T9G12.

**Construction of a 2-Site ELISA for Detection of proCPU**

MA-T12D11 or MA-T32F6 was diluted to 4 \(\mu\)g/mL in PBS (140 mmol/L NaCl, 2.7 mmol/L KCl, 8 mmol/L Na\(_2\)HPO\(_4\), and 1.5 mmol/L KH\(_2\)PO\(_4\), pH 7.4). Two hundred-microliter samples of these solutions were incubated at 4°C for 72 hours in the wells of polystyrene microtiter plates (Costar). The plates were emptied by shaking, and the wells were treated for 2 hours at room temperature with 200 \(\mu\)L PBS containing 10 g/L BSA. Then the wells were washed with PBS containing 0.002% Tween 80 (PBS-Tween) and finally with 200 \(\mu\)L of a solution containing 100 g mannitol and 20 g saccharose per liter. The plates were stored at −20°C. Immediately before use, plates were washed once with PBS-Tween. Plasma samples were diluted 1:20 (followed by serial 2-fold dilutions) in PBS containing Tween 80 (0.002%), BSA (1 g/L) (PTA buffer), and EDTA (5 mmol/L), and 180-\(\mu\)L samples were applied to the wells. After incubation for approximately 18 hours at 4°C in a moist chamber, the wells were washed with PBS-Tween. The plates were filled with 170-\(\mu\)L samples of MA-T28G6-HRP (diluted 1:4000) in PTA for MA-T12D11 and with 170-\(\mu\)L samples of MA-T9G12-HRP (diluted 1:4000) in PTA for MA-T32F6 and incubated for 2 hours at room temperature. After washing of the plates, the peroxidase reaction was performed by addition of 160-\(\mu\)L aliquots of a 0.1 mol/L citrate-0.2 mol/L sodium phosphate buffer, pH 5.0, containing 400 \(\mu\)g/mL o-phenylenediamine and 0.003% hydrogen peroxide to the wells. After 10 to 30 minutes at room temperature, the reaction was stopped with 50 \(\mu\)L of 4 mol/L H\(_2\)SO\(_4\). The absorbance was measured at 492 nm with an EL808 Ultra Microplate Reader (Bio-Tek instruments Inc). Calibration was performed with proCPU purified from plasma.

**Other proCPU Immunoassays**

The following available proCPU immunoassays were used: the commercially available assay from Milan Analytica using a sheep anti-proCPU polyclonal for coating as well as for detection; an ELISA in which MA-5081 (obtained from Hematologic Technologies Inc) was used for coating and a sheep anti-proCPU polyclonal for detection (Calbiochem); and an ELISA in which MA-5026 (obtained from Hematologic Technologies Inc) was used for coating and a sheep anti-proCPU polyclonal for detection (Calbiochem). In all 3 ELISAs, the values were expressed as percentage versus normal plasma.

**Construction and Production of proCPU Variants**

The cDNA of proCPU (provided by Dr M. Nesheim, Queens University, Canada), as reported by Eaton et al\(^8\) (except for A at position 291 in the cDNA of proCPU that was mutated into a T resulting in the same amino residue ie, Asp at position 75), was cloned into pcDNA3.1+ (Invitrogen) using the following primers:
GAATTCGATCTGGTACCTATGAAAGCTTTTG, creating a KpnI site, and CCGAGGCGAATGCTAGATCGAGGCC, creating a XhoI site. This cDNA sequence encodes for proCPU harboring Thr at positions 147 and 325. 

Thr at position 325 was mutated either in an Ile (using the Quickchange method [Stratagene] and the following primers: GC-TATTGAGAAATGATGAATACCGG and CCTGG-TATTTTTACTATTTTCTCAGTGC or in an Ala (using the following primers: GCTATTGAGAAATGCTAGAATACCGG and CCTGGATATTTTACTATTTCTCAATAGC). Thr at position 147 was mutated in an Ala using the following primers: GAGAAGAACACGACGCAAATAGCC and GGCATTITG-GCTTGTTCCTTCC.

The proCPU variants were transiently transfected in HEK293T cells using the Lipofectamine 2000 protocol (Invitrogen). Media were harvested 96 hours after transfection.

Testing the Reactivity of the Anti-proCPU MA for the Different proCPU Variants Using Immunoprecipitation Followed by Western Blotting

To test the reactivity of different anti-proCPU MA for the different proCPU forms (ie, proCPU-Thr147Thr325, proCPU-Thr147Ile325, proCPU-Thr147Ala325, proCPU-Ala147Thr325, and proCPU-Ala147Ile325), 2.5 mL of transfected medium was incubated overnight at 4°C with 100 µL of sepharose beads, to which MA-T27G4 (directed against proCPU) was coupled. After incubation, the beads were washed with PBS, and 100 µL of PBS containing 1% SDS was added to the beads followed by heating during 30 seconds at 100°C. A Sodium Dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the immunoprecipitated samples together with proCPU purified from plasma as a positive control was performed. Subsequently, the membranes were incubated with a secondary HRP-conjugated goat anti-mouse antibody (diluted 1:2000 in TBS) for 40 minutes and washed extensively with TBS. Subsequently, the membranes were incubated with a secondary HRP-conjugated goat anti-mouse antibody (diluted 1:2000 in TBS) for 40 minutes and washed extensively with TBS. The membranes were developed using the ECL detection system (Amersham Biosciences).

Testing the Reactivity of the Anti-proCPU Monoclonal Antibodies for Different proCPU Fragments

To samples containing proCPU (200 µg/mL) digested with thrombin/thrombomodulin, SDS was added to a final concentration of 1% followed by heating during 30 seconds at 100°C. The different proCPU fragments were separated by SDS-PAGE. Subsequently, the samples were transferred to a nitrocellulose membrane (Schleicher & Schuell), and nonspecific binding sites were blocked for 1 hour with 5% nonfat milk in Tris-buffered saline (TBS) for 1 hour with 5% nonfat milk in Tris-buffered saline (TBS). Membranes were incubated for 1 hour with primary antibodies (MA-T9G12, MA-T12D11, or MA-T28G6 at 30 µg/mL in TBS) raised against human, plasma-derived proCPU and washed extensively with TBS. Subsequently, the membranes were incubated with a secondary HRP-conjugated goat anti-mouse antibody (diluted 1:2000 in TBS) for 40 minutes and were washed extensively with TBS. The membranes were used for the Western blotting experiments, 144 MA combinations were selected and conjugated to HRP.

Results

Generation of Monoclonal Antibodies

Three fusions using spleen cells from Balb/c mice immunized with proCPU yielded 91 hybridomas. Based on their yield of production and purification, monoclonal antibodies were selected and conjugated to HRP.

Reactivity of Monoclonal Antibodies Toward Various proCPU Fragments

Twenty-seven monoclonal antibodies were selected for evaluation of their reactivity toward proCPU fragments generated on digestion of proCPU with thrombin/thrombomodulin. Different reactivities toward blotted proCPU fragments were observed between the monoclonal antibodies, subsequently showing distinction into 5 groups: (1) reacting with a 55-kDa proCPU fragment (eg, MA-T9G12; MA-T12D11); (2) reacting with a 55- and 35-kDa proCPU fragment; (3) reacting with a 55-, 35-, and a 25-kDa proCPU fragment; (4) reacting with a 55- and 35-kDa proCPU fragment; (5) reacting with a 55-, 44-, and 35-kDa proCPU fragment (eg, MA-T32F6); and (5) weakly reacting with blotted proCPU (eg, MA-T28G6).

Detection of proCPU and Thrombin/Thrombomodulin-Generated Fragments Thereof in a Sandwich-Type ELISA

Based on the differential reactivities of the MA in the Western blotting experiments, 144 MA combinations were tested in sandwich-type ELISAs for the detection of proCPU and thrombin/thrombomodulin-generated fragments thereof. Subsequently, based on the sensitivity, suitability for analysis of biological samples and specificity of the response, 2
TABLE 1. ProCPU Levels* According to the Genotype at Position 325, Measured With Two Different ELISA Combinations

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T12D11/T28G6-HRP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile/Ile</td>
<td>9.4±2.8</td>
<td>7.8±1.2</td>
</tr>
<tr>
<td>Ile/Thr</td>
<td>9.6±2.5</td>
<td>7.7±1.6</td>
</tr>
<tr>
<td>Thr/Thr</td>
<td>10.8±3.6</td>
<td>8.1±1.6</td>
</tr>
<tr>
<td>Overall</td>
<td>10.1±3.1</td>
<td>7.9±1.6</td>
</tr>
<tr>
<td><strong>T32F6/T9G12-HRP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile/Ile</td>
<td>0.0±0.0</td>
<td>0.0±0.1</td>
</tr>
<tr>
<td>Ile/Thr</td>
<td>4.2±1.7</td>
<td>4.2±1.2</td>
</tr>
<tr>
<td>Thr/Thr</td>
<td>7.3±2.9</td>
<td>8.4±3.8</td>
</tr>
<tr>
<td>Overall</td>
<td>5.4±3.0</td>
<td>6.3±3.8</td>
</tr>
</tbody>
</table>

*Expressed as μg/mL (mean±SD).

Table of proCPU levels according to the genotype at position 325, measured with two different ELISA combinations (ie, T12D11/T28G6-HRP and T32F6/T9G12-HRP) exhibiting distinct reactivities were selected for measurement of proCPU in plasma obtained from different proCPU genotypes. Whereas both ELISAs detect intact proCPU, digested proCPU is not or is very weakly recognized.

Measurement of proCPU in Biological Samples

In both ELISAs, a linear dose-response curve was observed between 500 and 7.8 ng/mL proCPU. Within the linear portion of these curves, correlation coefficients exceeded 0.98.

Addition of proCPU at a final concentration of 2.5, and 10 μg/mL to proCPU-depleted plasma (cfr methods) revealed recoveries in the T12D11/T28G6-HRP ELISA of 94±5.3%, 84±8.3%, and 90±0.2%, respectively, and in the T32F6/T9G12-HRP ELISA of 89±2.8%, 83±4.1%, and 74±4.3%, respectively.

The assay variability of the ELISAs was evaluated using 4 different plasma samples each assayed 4 times on 4 occasions. The intra-assay and interassay coefficients of variation were 9.6% and 14% for T12D11/T28G6-HRP and 6.3% and 6.0% for the T32F6/T9G12-HRP.

The interdilution coefficients of variation were 6% and 9.0% for T12D11/T28G6-HRP and T32F6/T9G12-HRP, respectively. ProCPU-depleted plasma (cfr methods) revealed no response in both ELISAs.

Measurement of proCPU in Plasma Obtained From Different Genotypes

One hundred seventy-two plasma samples that were characterized for their proCPU genotype at position 325 were obtained from the HIFMECH study (ie, 86 samples of the patient group and 86 samples of the control group) (cfr materials). The proCPU levels of these 2 groups yielded values of 4.3 to 22.1 μg/mL in the T12D11/T28G6-HRP ELISA and 0 to 17.6 μg/mL in the T32F6/T9G12-HRP ELISA.

However, grouping according to the 325 genotype (Table 1) demonstrated that T12D11/T28G6-HRP was insensitive to this polymorphism (eg, for the control group, 9.4±2.8, 9.6±2.5, and 10.8±3.6 μg/mL for the Ile/Ile, Ile/Thr, and Thr/Thr isoforms, respectively). Strikingly, T32F6/T9G12-HRP revealed a complete lack of reactivity with the Ile/Ile genotype (eg, for the control group, 0.0±0.0, 4.2±1.7, and 7.3±2.9 μg/mL for the Ile/Ile, Ile/Thr, and Thr/Thr isoforms, respectively).

Analysis of these samples by commercially available assays (Table 2) revealed antigen values between 44% to 86%, 68% to 88%, and 100% to 140% versus control plasma for the Ile/Ile, Ile/Thr, and Thr/Thr isoforms, respectively.

Reactivity of the Different Anti-proCPU Monoclonal Antibodies Toward proCPU Variants

To additionally investigate the lack of reactivity of T32F6/T9G12-HRP for the Ile/Ile 325 genotype, the reactivities of MA-T9G12, MA-T12D11, and MA-T32F6 were tested toward 5 recombinantly expressed proCPU variants, ie, proCPU-Thr147Thr325, proCPU-Thr147Ile325, proCPU-Ala147Thr325, proCPU-Ala147Ile325, and proCPU-Thr147Ala325 in a Western blot analysis.

The blotting experiments revealed that MA-T9G12 and MA-T12D11 reacted with all recombinantly expressed proCPU variants. Strikingly, MA-T32F6 did react with proCPU purified from plasma and recombinantly expressed proCPU-Thr147Thr325 and proCPU-Ala147Thr325 but not with the recombinantly expressed proCPU-Thr147Ile325, proCPU-Ala147Ile325, and proCPU-Thr147Ala325 variants.

SDS-PAGE analysis of the O-deglycosylated proCPU-Thr147Thr325 and proCPU-Ala147Thr325 variants showed for both variants a lower molecular weight compared with the nondeglycosylated variants. Western blot analysis revealed that MA-T32F6 is still able to react with the O-deglycosylated proCPU-Thr147Thr325 and proCPU-Ala147Thr325 variants.

Detection of Recombinantly Expressed proCPU Variants in the Different proCPU ELISAs

The data represented in Table 3 demonstrate that the 5 proCPU variants react equally in the T12D11/T28G6-HRP...
ELISA, whereas neither the proCPU-Ile325 variants nor the proCPU-Thr147Ala325 variant revealed any reactivity in the T32F6/T9G12-HRP ELISA.

**Correlation With CPU Activity**

The correlation of the proCPU antigen concentration with the CPU activity was calculated for 48 genotyped samples (Table 4). Good correlations for the overall as well as for the 325 genotyped groups were observed for T12D11/T28G6-HRP, whereas for T32F6/T9G12-HRP, a significant correlation between antigen and activity was found in the ThrThr subgroup only.

**Discussion**

For the measurement of proCPU concentrations in human plasma, both functional and immunological assays are available. The major advantage of immunological assays is that no activation of the zymogen is required, which makes ELISAs easy to perform. However, studies investigating a possible relationship between proCPU levels and cardiovascular diseases revealed conflicting results. ProCPU levels above the 90th percentile of population-based controls increased the risk for venous thrombosis compared with proCPU levels below the 90th percentile. In a case-control study of patients with stable angina pectoris, the plasma proCPU level in patients was significantly higher than in controls. In contrast, another study concluded that high proCPU levels in these samples. Obviously, this has to be taken into account for any analysis on the association between proCPU levels and cardiovascular pathologies. The importance of this phenomenon is even more stressed because the most stable variant, exhibiting a more pronounced antifibrinolytic effect, is being underestimated. Therefore, when the antigen levels were grouped according to the 147 genotype, all apparent 147 genotype–based differences were solely attributable to the association with the 325 genotype consequent to the strong linkage disequilibrium between both genotypes. This observation forces a reconsideration of conclusions made in earlier reports before the identification of the polymorphism at position 325, suggesting an association between the 147 genotype and antigen levels.

**Recombinant expression of the 4 isoforms**

In this study, we describe the development of 2 ELISAs (T12D11/T28G6-HRP and T32F6/T9G12-HRP) for measurement of proCPU in plasma. When the antigen values obtained with these ELISAs were grouped according to the 325 genotype, T12D11/T28G6-HRP revealed to be independent of the 325 genotype. Strikingly, T32F6/T9G12-HRP revealed a complete lack of reactivity with the Ile/Ile genotype. This was observed both in the plasma of healthy subjects and in that of patients with myocardial infarction. Furthermore, the antigen values of the Ile/Ile genotype–harboring samples in T32F6/T9G12-HRP would be caused by interference of plasma proteins (eg, plasminogen and fibrinogen), affecting exclusively the interaction between proCPU-Ile325 and MA-T32F6. Thus, O-linked glycosylation of Thr325 might be required for recognition by MA-T32F6, whereas neither the proCPU-Ile325 variants nor the proCPU-Thr147Ala325 variant did not react in the T32F6/T9G12-HRP, nor did MA-T32F6 react with this proCPU variant in a Western blotting experiment, providing additional evidence that Thr at position 325 is part of the epitope of MA-T32F6. Taken together, these observations also exclude any indirect effect, ie, the possibility that the lack of reactivity of Ile/Ile genotyped samples in T32F6/T9G12-HRP would be caused by interference of plasma proteins (eg, plasminogen and fibrinogen), affecting exclusively the interaction between proCPU-Ile325 variants and MA-T32F6.

**On the other hand**, Boffa et al demonstrated that all 4 N-linked glycosylation sites (ie, Asn-22, Asn-51, Asn-63, and Asn-86) in proCPU are utilized and suggested a heterogeneity in usage of O-linked glycosylation sites. Thus, O-linked glycosylation of Thr325 might be required for recognition by MA-T32F6, resulting in a lack of reactivity of proCPU-Ile325 variants. However, this could be excluded by deglycosylation experiments demonstrating that the putative presence of sugar moieties at Thr325 does not affect the epitope of MA-T32F6.

Most of the presently commercially available antigen assays seemed to be partially dependent on the 325 genotype, indicating that previous data showing an association between Thr/Thr polymorphism and apparently higher antigen levels are the consequence of differential immunological reactivities of the isoforms in these ELISAs. Importantly, underestimation of the Ile/Ile in these commercially available antigen assays will result in erroneous conclusions with regard to proCPU levels in these samples. Obviously, this has to be taken into account for any analysis on the association between proCPU levels and cardiovascular pathologies. The importance of this phenomenon is even more stressed because the most stable variant, exhibiting a more pronounced antifibrinolytic effect, is being underestimated. Therefore,
the conclusions of previous studies evaluating an association between proCPU antigen levels and genotypes in relation to cardiovascular diseases\textsuperscript{19,20,22,33} should be reconsidered.

In conclusion, our data demonstrate that great care should be taken when evaluating proCPU antigen values as a putative causative agent or as a diagnostic risk marker for cardiovascular events. Immunological assays for proCPU antigen determination used in large epidemiological studies should be very well characterized with respect to genotype-dependent reactivity.

From the data obtained in this study, we can also conclude that T12D11/T28G6-HRP allows to quantify unambiguously (ie, genotype-independent) proCPU antigen levels whereas T32P6/T9G12-HRP constitutes an easy, immunological method to identify patients homozygous for Ile325.

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