Inactivation of a MAPK-like protein kinase and activation of a MBP kinase in germinating barley embryos

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Abstract We provide evidence for involvement of two different 45 kDa protein kinases in rehydration and germination of barley embryos. In dry embryos, a myelin basic protein (MBP) phosphorylating kinase was detected, which could be immunoprecipitated with an anti-MAPK (mitogen-activated protein kinase) antibody. Rehydration of the embryo induced a decrease in activity of this 45 kDa MAPK-like protein kinase. In addition, activity of a MBP kinase of the same molecular weight was subsequently found to be induced. This second MBP kinase activity could not be immunoprecipitated with the anti-MAPK antibody and was induced only in germinating embryos, not in dormant embryos.

Key words: Mitogen-activated protein kinase; Myelin basic protein kinase; Germination; Rehydration; *Hordeum distichum*

1. Introduction

Seed germination is a process that depends on both internal and environmental factors, such as plant hormone levels, light and temperature [1]. The germination process is a continuation of seed development, in which the embryo is formed but further growth is arrested, as the process is stopped by desiccation of the seed. Under appropriate conditions, mature desiccated seeds are rehydrated, metabolism is resumed and seeds start to germinate. When intact viable seeds do not germinate under favorable conditions, they are called dormant [2].

For barley grains, two of the key factors that determine whether an embryo will germinate are the levels of abscisic acid (ABA) and gibberellins. ABA is an important regulator of dormancy; it is involved both in maturation of the seed and in inhibition of the germination process by antagonizing gibberellin activity during germination [3]. Dormancy of barley grains can be broken by several agents; some of these affect the ABA content of the embryos while others, such as the fungal toxin fusicoccin, probably interfere with ABA signal transduction [4]. Several studies have shown the involvement of protein phosphorylation in ABA signal transduction in seeds (reviewed by Heimovaara-Dijkstra et al. [5]). In wheat embryos and barley aleurone, mRNA levels of the serine/threonine kinase PKABA1 are induced by ABA [6,7]. Furthermore, ABA has been shown to induce the activity of a mitogen-activated protein kinase (MAPK) in barley aleurone protoplasts [8].

MAPKs form a family of protein kinases that are activated in response to various extracellular stimuli in eukaryotic organisms [9,10]. In plants, MAPKs have been reported to be activated by different types of stress, such as cold, drought, wounding, and salt stress [11–14]. In addition, plant MAPKs can be activated by hydration [15], elicitors [16,17] and several plant hormones, including salicylic acid [18], ethylene [19] and ABA [8]. MAPKs also appear to be involved in cell cycle regulation [20] and auxin signal transduction [21]. These results demonstrate that MAPKs play a role in stress signalling as well as in cell cycle regulation.

Since several of the stimuli that influence MAPK activity are also known to be involved in germination (i.e. stress, ABA and cell division), we investigated MAPK activity in germinating barley embryos. In particular, the activation of MAPK by ABA in barley aleurone protoplasts raised the question whether a MAPK pathway would be involved in ABA-induced inhibition of germination. However, we did not find an effect of ABA on MAPK activity in barley embryos. Instead, we found that MAPK-like activity in barley embryos is regulated post-translationally in a hydration-dependent way. We show that upon rehydration of barley grains, the activity of a 45 kDa MAPK-like protein kinase decreases. A subsequent increase in 45 kDa protein kinase activity was observed only in germinating embryos, and not in dormant embryos. This second kinase activity has different properties than the rehydration-inactivated MAPK-like kinase.

2. Materials and methods

2.1. Plant material

Non-dormant *Hordeum distichum* L. cv. Triumph grains were from Heineken Technical Services (HTS) (Zoeterwoude, The Netherlands). Dormant grains were obtained by growing genetically identical Triumph plants first 16 h at 21°C/8 h at 10°C under continuous light for 20 days. Then conditions were changed to 16 h at 14°C/8 h at 10°C under continuous light until the grains were fully ripe. Grains were dried to 6–8% moisture content and stored at −20°C to preserve dormancy.

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Abbreviations: ABA, abscisic acid; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein

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2.2. Germination conditions

Ten–20 intact grains were transferred to two layers of Whatman No. 1 paper (Whatman, UK) in a Petri dish (9 cm) containing 3 ml distilled water. Plates were sealed with Parafilm to prevent evaporation and incubated in the dark at 20°C. Grains were scored as germinated when the radicle was ≥1 mm. Water uptake of dormant and non-dormant grains was determined under the same conditions by weighing each grain at designated times.

2.3. Protein extraction

Embryos were dissected carefully from barley grains and were immediately frozen in liquid N_2. Ten embryos were ground in 1 ml of 20 mM Tris, pH 8.0, 40 mM $Na_4P_2O_7$, 50 mM NaF, 5 mM $MgCl_2$, 0.1 mM Na_3VO_4, 10 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and one tablet of complete protease inhibitors per 150 ml (Boehringer). The suspension was left on ice for 15 min and centrifuged at $15\,000\times g$ for 5 min at 4°C. The supernatant was stored at −20°C for biochemical analysis.

2.4. Immunoprecipitation

For immunoprecipitation (basically according to Knetsch et al. [8]), 200 μg of total protein extract was added to 10 μl anti-ERK1 antibody (K-23, rabbit polyclonal sc-94, raised against a peptide which corresponds to amino acids 305–327 mapping within subdomain XI of ERK1-encoded MAP kinase p44 of rat origin, Santa Cruz Biotechnology) that was precoupled to 20 μl protein G-Sepharose 4B-fast flow (Pharmacia). The immunoprecipitate was washed and resuspended in sample buffer (60 mM Tris, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue) to analyze in an in-gel kinase assay.

2.5. In-gel protein kinase assay

In-gel kinase assays were performed as described by [22]. Embryo protein extracts (30 μg) or immunoprecipitates were separated on a 12.5% (w/v) SDS polyacrylamide gel containing 0.5 mg/ml myelin basic protein (MBP). The gel was fixed in 20% isopropanol, 50 mM Tris, pH 8.0, for 30 min, pretreated in 50 mM Tris, pH 8.0, 5 mM 2-mercaptoethanol (buffer A) for 60 min and denatured in 6 M guanidine–HCl in buffer A. The proteins were renatured overnight in 0.04% Tween-40 in buffer A at 4°C. The gel was incubated first in buffer B (40 mM HEPES, pH 8.0, 2 mM dithiothreitol, 0.1 mM EGTA, 5 mM $MgCl_2$) for 60 min and next in 50 μM ATP, 125 μCi/ml [γ-^{32}P]ATP in buffer B for 60 min. The gel was washed six times for 15 min each in 5% TCA, 1% NaPP, dried and exposed to film.

2.6. Western blot analysis

Embryo protein in sample buffer was separated by 12.5% (w/v) SDS–PAGE and blotted to nitrocellulose. Immunoblots were incubated with anti-ERK1 antibody (1:1000) at 4°C. Polypeptides were visualized by incubation in horseradish peroxidase-labeled goat anti-rabbit antibodies, followed by enhanced chemiluminescence detection (ECL, Amersham).

3. Results

3.1. MBP kinase activity in barley embryos during germination of intact grains

To identify MAPK activity in germinating barley embryos, we used an in-gel kinase assay to measure phosphorylation of MBP, which is a known substrate of MAPKs. Intact grains were imbibed (hydrated) and, at designated times, protein extracts were made of carefully dissected embryos. In an in-gel assay (Fig. 1A), a dominant 45 kDa MBP kinase activity was detected that showed different activation levels during imbibition. Two additional MBP phosphorylating proteins migrated at values of approximately 80 and 60 kDa, but these activities

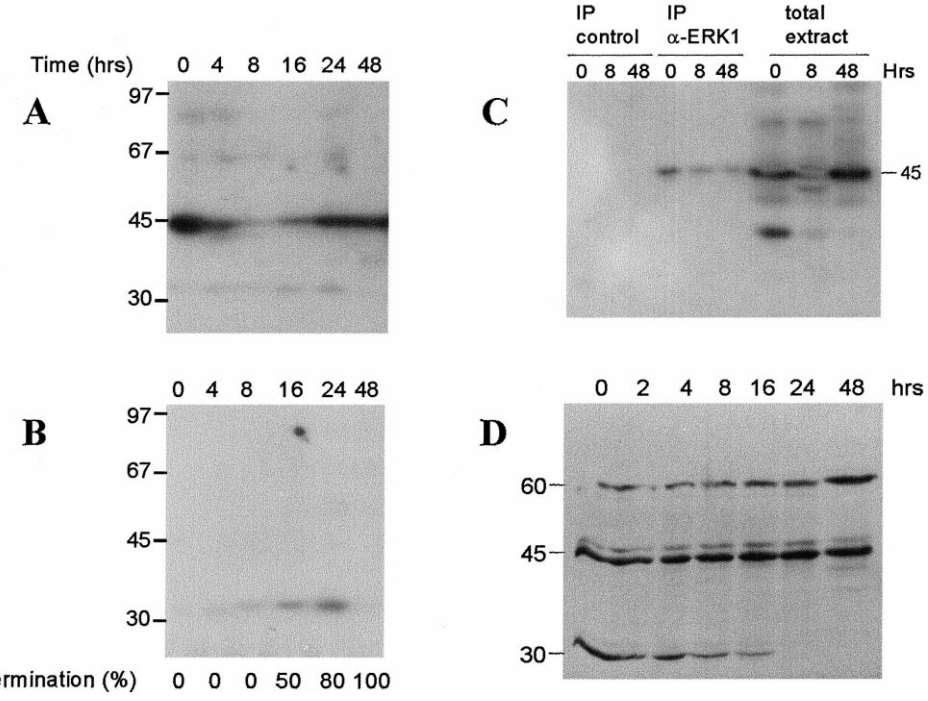

Fig. 1. Activity of MBP phosphorylating kinases and expression of MAP kinases in barley embryos. (A) In-gel MBP kinase assay of embryo extracts. Intact barley grains were imbibed in water and embryos were dissected at designated times. 30 μg of protein was loaded on the gel. (B) Control gel without MBP. Samples are identical to samples loaded in (A). (C) Immunoprecipitation followed by in-gel kinase assay. Protein extracts of dry embryos and 8 h and 48 h imbibed embryos (200 μg) were used for immunoprecipitation with anti-ERK1 antibody. Immunocomplexes were analyzed for MBP phosphorylation by in-gel kinase assay. Lanes 1–3: control IP using only protein G, no antibody. Lanes 4–6: immunoprecipitations with protein G and anti-ERK1 antibody. Lanes 7–9: total extracts 20 μg. One representative example of three independent experiments is presented. (D) Western analysis of barley embryo extracts with anti-ERK1 antibody. 20 μg of protein of each sample was loaded.

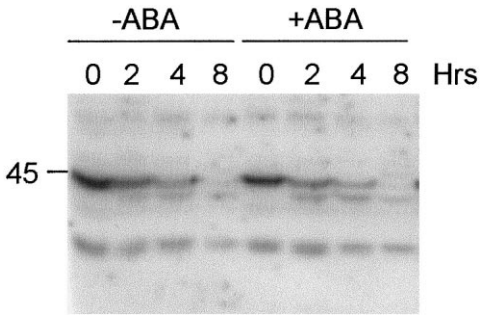

Fig. 2. Effect of ABA on MBP phosphorylating activity in embryos during imbibition of barley grains. Intact grains were treated with 100 μM ABA, embryos were isolated at designated times and in-gel kinase analysis was performed as described for Fig. 1A. 30 μg of protein was loaded. One representative example of four independent experiments is presented.

were weak and not reproducible. A 38 kDa band was also present in the control gel without MBP (Fig. 1B) and was, therefore, judged to result from autophosphorylation.

The 45 kDa MBP phosphorylating kinase isolated from dry embryos appeared to be highly active. Upon hydration, this activity decreased within 8 h to a minimum of 20% of the initial level of activity. After 16 h, the activity started to increase again, reaching almost its initial activation level after 48 h of imbibition. This increase in MBP kinase activity coincided with visible germination of the embryo (Fig. 1A).

The molecular mass of the 45 kDa protein kinase and its ability to use MBP as a substrate strongly suggested that this kinase is a MAPK. This hypothesis was tested by the use of an anti-MAPK antibody raised against rat ERK1. MBP kinase activities of anti-ERK1 immunoprecipitates of extracts of 0, 8 and 48 h imbibed embryos were analyzed in an in-gel assay. Fig. 1C shows that only the activity present in dry embryos could be immunoprecipitated (lane IP α-ERK1 0 h). This immunoprecipitated kinase appeared to have a molecular mass similar to that of the kinase in the total protein extract. The germination-related increase in kinase activity was not precipitated by the anti-ERK1 antibody (lane IP α-ERK1 48 h), thus indicating that the decrease and subsequent increase in 45 kDa kinase activity during imbibition are caused by different kinases. These kinases will be further referred to as MAPK-like kinase and MBP kinase. No MBP phosphorylating activity was precipitated in the controls without anti-ERK1 antibody (lanes IP control).

Expression of barley MAPKs during germination was studied by Western analysis using the anti-ERK1 antibody. At a value of approximately 45 kDa, two polypeptides were detected by the anti-ERK1 antibody (Fig. 1D), the smaller of which presumably corresponds to the MAPK-like kinase, as judged from its position in the gel. Two-dimensional Western analysis of embryo protein extracts revealed only one anti-ERK1 cross-reacting spot for each of these positions (data not shown). In addition, two other polypeptides were detected by the anti-ERK1 antibody (Fig. 1D); one represented by a 30 kDa band, and another with a molecular mass of approximately 60 kDa. Abundance of the two 45 kDa anti-ERK1-detected kinases remained constant during the first 48 h of imbibition. Therefore, we conclude that the inactivation of the MAPK-like 45 kDa kinase during imbibition was not caused by differential expression of the protein.

3.2. Effect of ABA on MAPK-like activity

As a 45 kDa MAPK from barley aleurone is known to be induced by ABA [5,8], we investigated whether the MAPK-like kinase activity in embryos was affected by ABA. The effect of ABA on the MAPK-like kinase activity was tested by imbibing intact grains in the presence of ABA. Since the barley cultivar used, Triumph, is not very sensitive to ABA, a high concentration of ABA (10^{-4} M) was used to delay germination. Even at this high concentration, no effect of ABA was seen on MBP kinase activity during the first 8 h of imbibition (Fig. 2).

3.3. MBP kinase activity in dormant embryos, and effect of dormancy-breaking agents

Next, we further investigated the regulation of the 45 kDa MAPK-like and MBP kinases during germination. In particular, we wanted to know whether the changes in kinase activation during germination were due to rehydration or related to germination. Therefore, MBP kinase activity was measured in embryos of dormant barley grains, which do not germinate, even under favorable conditions. The dormant grains used were genetically identical to the non-dormant grains; dormancy was induced environmentally [23]. Fig. 3A shows

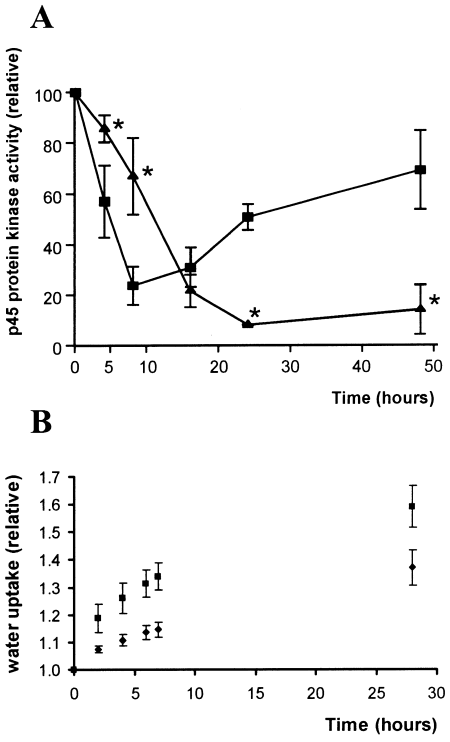

Fig. 3. MBP kinase activity and water uptake in non-dormant and dormant barley grains. (A) In-gel MBP kinase analysis as described in Fig. 1A. ■, non-dormant grains; ▲, dormant grains. Activity of the 45 kDa MBP phosphorylating activity (p45 protein kinase) was quantified using ImageQuant (Molecular Dynamics). Data are the mean of three independent experiments. (*) indicates significant difference with non-dormant grains, as determined with the Student's *t*-test ($P < 0.05$). (B) Water uptake of intact non-dormant (■) and dormant (♦) barley grains. Water uptake was determined by weighing individual grains repeatedly during imbibition. Two independent experiments with in total 20 non-dormant and 20 dormant grains are presented. The mean values ± S.D. of 20 individual grains are shown.

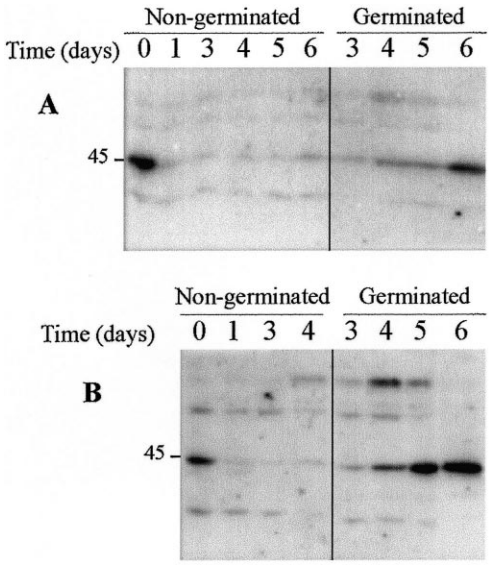

Fig. 4. Correlation of activation of MBP kinase with germination. Dormant grains were incubated in 10^{-4} M fusicoccin (A), or 1% ethanol (B). Each day, germinated grains were separated from non-germinated grains and embryos were assayed separately for MBP kinase activity. 30 μg of total protein was loaded.

45 kDa MBP kinase activity in dormant and non-dormant barley embryos. In dormant embryos, the initial level of kinase activity was similar to that in non-dormant embryos. Upon imbibition, the MAPK-like activity decreased, although not as fast as in non-dormant embryos. No activation of the MBP kinase was seen in dormant embryos, which indicates that activation of the MBP kinase during imbibition is correlated with germination.

As MAPK-like activity decreased in embryos of both non-dormant and dormant grains, this decrease appears to be a direct or indirect effect of rehydration of the grain, which occurs both in dormant and non-dormant grains that are placed in water. In fact, water uptake of intact dormant grains appeared to be slower than for non-dormant grains (Fig. 3B). The same effect was observed when water uptake rate of only the embryos was measured (data not shown). This difference in water uptake rate is consistent with the slightly slower decrease of MAPK-like kinase activity in embryos of dormant grains.

To study regulation of the MBP kinase during germination, dormant grains were treated with different dormancy-breaking agents and assayed for MBP protein kinase activity. Dormant grains were treated with either fusicoccin (Fig. 4A) or ethanol (Fig. 4B), which both induce germination between 3–7 days. At fixed intervals, germinated and non-germinated embryos were separated and assayed separately for MBP kinase activity. Fig. 4A,B shows that MBP kinase activity was activated only in grains that had germinated. As all grains had developed under the same, dormancy-inducing conditions and had been given the same treatment, the difference in MBP kinase activity cannot have been caused by a difference in applied growth conditions during seed development, or have been a direct effect of the compound used. Therefore, this experiment shows the strict correlation of increased MBP kinase activity with germination.

4. Discussion

In this study, we show that the germination process of barley is associated with a decrease in MAPK-like protein kinase activity and a subsequent increase in MBP kinase activity. Using an in-gel assay, two different MBP phosphorylating kinases with the same molecular mass (45 kDa) were identified. The initial MBP phosphorylating activity, which decreased upon rehydration of the grain, could be immunoprecipitated using an anti-rat ERK1 antibody, consistent with the hypothesis that this kinase is a MAPK. However, the following increase in MBP phosphorylating activity during germination could not be immunoprecipitated with the same antibody, suggesting that this activity is due to another class of MBP kinase.

The decrease in MAPK-like activity could be observed during imbibition of both dormant grains (which do not germinate) and non-dormant grains (Fig. 3A). In addition, the slower decrease in kinase activity in embryos of dormant grains corresponded with the lower rate of water uptake of these grains (Fig. 3B). Therefore, we conclude that the decrease of 45 kDa MAPK-like kinase activity in embryos of imbibed grains is induced upon rehydration of the grain, but is not related to germination. To our knowledge, this is the first report to imply MAPK-like activity in seed imbibition.

As judged from the results presented here, we have no indications that ABA can induce MAPK activity in germinating barley embryos. Treatment of barley grains with 100 μM ABA did not induce MAPK-like kinase activity, as measured with an in-gel assay (Fig. 2). Also when isolated embryos were treated with ABA for short periods, no difference in MBP kinase activity was observed, compared to a water control (C. Testerink and M. Vennik, unpublished results). Although ABA is able to induce responses in diverse tissues, these responses and the corresponding signal transduction pathways are often tissue-specific [5]. Therefore, it is not surprising that embryo cells do not display the same MAPK activity as do aleurone protoplasts in response to ABA. However, it is also possible that ABA does induce MAPK activity in embryos, but that this activity cannot be detected. MAPK activity in aleurone protoplasts peaks after 3 min of ABA treatment and returns to basal level within 10 min [8]. Since an embryo consists of a large number of cells, a signal, such as ABA, will not reach all the cells at the same time, like it does in a protoplast suspension. Therefore, these kinds of fast responses are not likely to be detected when treating intact embryos or grains.

The MBP kinase activities during embryo germination have remarkably long-term kinetics. The inactivation of the MAPK-like kinase in barley embryos is very slow as opposed to the rapid and transient activation in minutes that has been reported in most other plant MAP kinase studies. In animal cells, sustained MAPK activation has been described for Rap1-mediated nerve growth factor-induced MAPK activation [24] and for JNK1 activation leading to apoptosis [25]. In tobacco cells, persistent MAPK activation has been observed in elicitor-treated suspension cells [26] and in salicylic acid-treated leaves [27].

After the decrease of MAPK-like activity, the activity of the other MBP kinase increased when embryos started to germinate. Using different methods to manipulate germination, we could show a clear correlation between germination and

45 kDa MBP kinase activity. In embryos of dormant grains, activity was not induced, but when dormancy was broken with ethanol or fusicoccin, induction of MBP kinase activity was observed in embryos that germinated, but not in those that did not germinate (Fig. 4). The difference in MBP kinase activity between dormant and non-dormant embryos could also have been caused by the differences in growth conditions of the seeds that are not related to germination behavior. However, this possibility can be excluded in the case of dormancy breakage with fusicoccin and ethanol. Therefore, we conclude that the increase in MBP protein kinase activity in barley embryos is strictly correlated with germination.

Interestingly, MBP kinase activity starts to increase shortly after radicle protrusion. Several proteins have been described, whose expression correlates with visible germination. Most of these proteins are enzymes, which are likely to be involved in normal cellular metabolism in the post-germinative stage [1]. The 45 kDa MBP kinase could be a possible signalling intermediate in the pathway leading to post-germination-related enzyme activity. Alternatively, it could play a role in induction of cell division, which is known to take place shortly after visible germination [1].

Our immunoprecipitation results suggest that the 45 kDa protein kinase activity is caused by two different kinases, one of which is a MAPK. As the germination-related MBP kinase is not precipitated by the α-ERK1 antibody, it could very well belong to a different class of protein kinases. MBP kinases that are not related to MAPKs have been identified before in plants [28,29]. However, at the moment we cannot exclude the possibility that the germination-related MBP kinase is another type of MAPK that is not recognized by the α-ERK1 antibody. Although unlikely, it is even possible that it is the same kinase as the rehydration-inactivated MAPK-like kinase, and that the lack of immunoprecipitation is caused by other factors that are different in germinated embryos and dry embryos, such as the presence of the kinase in a complex or modification of the kinase itself. Only the purification of the 45 kDa MBP kinases and determination of the protein sequences can conclusively answer this question. In conclusion, a rehydration-inactivated kinase, that has several properties of a MAPK, has been identified in barley embryos. In addition, a MBP kinase of the same molecular weight is activated upon germination of barley grains. Further characterization should reveal the identity of this kinase.

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