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Subcellular differences in post-translational modification of barley 14-3-3 proteins

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Abstract Expression and post-translational modification of barley 14-3-3 isoforms, 14-3-3A, 14-3-3B and 14-3-3C, were investigated using isoform-specific antibodies. Although all three isoforms were shown to be present in the cytosolic, the nuclear and the microsomal cell fractions, differences in post-translational modification were identified for the different cell fractions. Germination-related modifications of 14-3-3 proteins were observed in the cytosol and the microsomal fraction, but not in the nucleus. In vitro proteolytic cleavage of 14-3-3 proteins using trypsin suggests that for 14-3-3A this change was caused by proteolytic cleavage of the unconserved C-terminal region.

Key words: 14-3-3 Protein; Post-translational modification; Cell compartment; Germination; *Hordeum distichum*

1. Introduction

14-3-3 Proteins are thought to be present in all eukaryotes, and have been shown to carry out a range of different functions. In general, 14-3-3 proteins interact with other proteins, playing a role either in signal transduction cascades or as direct modulators of enzyme activity [1,2]. In plants, 14-3-3 proteins were shown to bind several targets in different cell compartments, such as the plasma membrane H^+-ATPase, the cytosolic enzymes nitrate reductase and sucrose phosphate synthase, and nuclear transcription factors [3–10]. In addition, 14-3-3 proteins form a complex with chloroplast precursor proteins and are present in the chloroplast stroma [11,12].

Since there is limited evidence for functional isoform-specificity of 14-3-3 proteins, tissue-specific and temporal regulation of 14-3-3 expression seem to be a major way to control 14-3-3 proteins. Tissue-specific expression of 14-3-3 proteins in plants has been described in *Arabidopsis* and in germinating barley embryos [13,14]. In addition, specific 14-3-3 isoforms have been shown to be induced upon different stimuli, such as elicitors, fusicoccin and cold treatment [15–17].

Little is known about subcellular localization of specific 14-3-3 isoforms. Compartmentalization of 14-3-3 proteins is likely to play an important role by sequestering signalling components to a specific cell compartment (reviewed in [18,19]). In *Arabidopsis*, specific 14-3-3 isoforms were shown to be present in the chloroplast stroma [11]. In addition, the nuclear fraction of cultured *Arabidopsis* suspension cells was shown to contain different amounts of certain 14-3-3 protein bands, compared to the total protein fraction [20]. However, since an antibody was used that cross-reacted with different 14-3-3 isoforms, it is not known whether these different bands in the nucleus represent different 14-3-3 isoforms or are due to differential post-translational modification of the same 14-3-3 isoforms. Changes in apparent molecular weight of 14-3-3 proteins, caused by modifications such as phosphorylation and Ca^{2+} binding, have been described for 14-3-3 proteins [21,22].

With use of a unique set of isoform-specific antibodies [14], we were able to test whether isoform-specificity exists in subcellular targeting of 14-3-3 isoforms 14-3-3A, 14-3-3B and 14-3-3C during germination of barley. Although all three isoforms were present in the nucleus, the cytosol and the microsomal fraction, subcellular differences in post-translational modification of the 14-3-3 isoforms were observed, which points at specific use of 14-3-3 isoforms at the cellular level.

2. Materials and methods

2.1. Plant material

Barley grains (*Hordeum distichum* L. cv. Triumph, harvested 1989) were obtained from Heineken Technical Service (HTS), The Netherlands. Dormant grains were produced in the phytotron according to [23]. The grains were germinated on two layers of Whatman No. 1 paper (Whatman, UK) in a Petri dish (9 cm) containing 3 ml distilled water at 25°C in the dark. After incubation, the embryos were dissected from the grains for protein extraction. In experiments using isolated embryos, 10 embryos from dry grains were incubated in a 24 wells plate containing 300 μl distilled water per well at 20°C in the dark.

2.2. Protein extraction and cell fractionation

Three types of solution were used: solution A: 10 mM Tris–HCl pH 7.5, 250 mM sucrose, 1 mM EDTA; solution B: 5 mM potassium phosphate pH 7.8, 250 mM sucrose, 4 mM KCl; solution C: 50 mM Tris–HCl pH 8.0, 500 mM NaCl, 5 mM EDTA, 20% (v/w) glycerol, 10 mM 2-mercaptoethanol. Complete protease inhibitors (Boehringer, Mannheim) and pepstatin 1 μg/ml were added to all solutions. The extracts in buffer A were passed through a nylon filter (45 μm) and were subsequently centrifuged at $1000\times g$ for 5 min. The $1000\times g$ pellet containing the nuclei was suspended in 200 μl solution C. The supernatant was centrifuged at $10\,000\times g$ and the resulting pellet, containing mitochondria and other organelles, was omitted. The $10\,000\times g$ supernatant was centrifuged at $100\,000\times g$. The $100\,000\times g$ pellet containing the microsomes was suspended in 200 μl solution B and the $100\,000\times g$ supernatant yielded the cytosolic fraction.

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2.3. Electrophoretic separation and Western analysis

Samples for protein analysis were prepared using sodium dodecyl sulfate (SDS) sample buffer and were separated on a 12% (w/v) SDS–PAGE gel [24]. The proteins were electrotransferred to a nitrocellulose membrane (Schleicher and Schuell). For Western analysis, specific antibodies [14] recognizing the separate barley 14-3-3 isoforms 14-3-3A, 14-3-3B and 14-3-3C were used. The commercial cross-reacting antibody anti-human 14-3-3β (K-19) was purchased from Santa Cruz. Visualization was performed with goat anti-rabbit horseradish peroxidase (GAR–HRP) conjugate (Promega) followed by chemoluminescent detection.

2.4. Protein assay and tryptic digestion

Partial digestion of 14-3-3 proteins with trypsin (Sigma, bovine pancreas) was carried out in 25 mM Tris pH 7.5, 5 mM $CaCl_2$ at 37°C. The trypsin concentration was 1/100 of the protein concentration for incubations with crude embryo extracts and 1/10 for incubation with *Escherichia coli*-expressed 14-3-3A, 14-3-3B and 14-3-3C. The reactions were stopped by addition of SDS sample buffer.

3. Results

3.1. Expression of 14-3-3A, 14-3-3B and 14-3-3C protein in cell fractions of germinating barley embryos

Using isoform-specific anti-14-3-3 antibodies, expression of the barley 14-3-3A, B and C isoforms was studied in different cell fractions of isolated embryos during germination. Fig. 1 shows that all three 14-3-3 isoforms were present in the nuclear, the microsomal and the cytosolic fraction, both in dry embryos (lanes N0, M0 and C0, respectively) and in germinated embryos (N44, M44 and C44). Interestingly, in the cytosol and microsomal fraction of germinated embryos, two 14-3-3A bands with an apparent molecular weight of 30 and 28 kDa were present, while in dry embryos, almost exclusively 30 kDa 14-3-3A was detected. The nuclear fraction, either from dry or germinated embryos, predominantly contained the 30 kDa 14-3-3A form. In addition, minor lower molecular weight bands were detected. The 14-3-3B and 14-3-3C isoforms had an apparent molecular weight of 31 kDa.

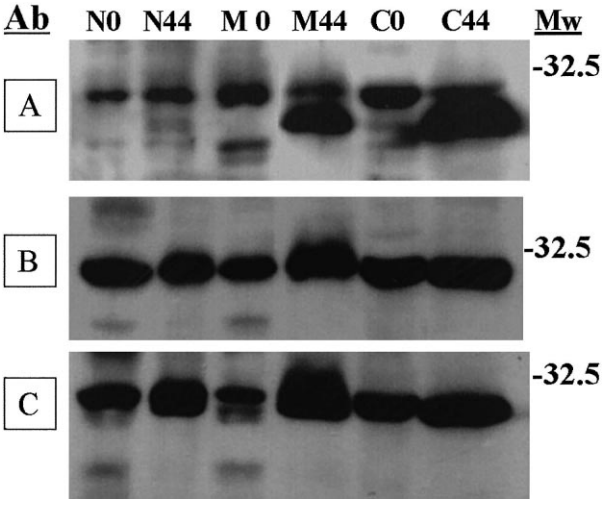

Fig. 1. Expression of 14-3-3 proteins in cell fractions of non-germinated and germinated barley embryos. Western analysis following SDS–PAGE, probed with specific antibodies (Ab) for the 14-3-3 isoforms (A: 14-3-3A, B: 14-3-3B and C: 14-3-3C) and a cross-reacting antibody (CR). Samples (15 μg protein) of the different fractions (C: cytosol, M: microsomal fraction, N: nuclear fraction) of ungerminated (0: dry embryo) and germinated (44: 44 h imbibed) embryos were loaded on a 12% gel. Immunodetection with separate antibodies was visualized using GAR–HRP conjugate in combination with chemoluminescent detection. Molecular weight markers (Mw) are indicated.

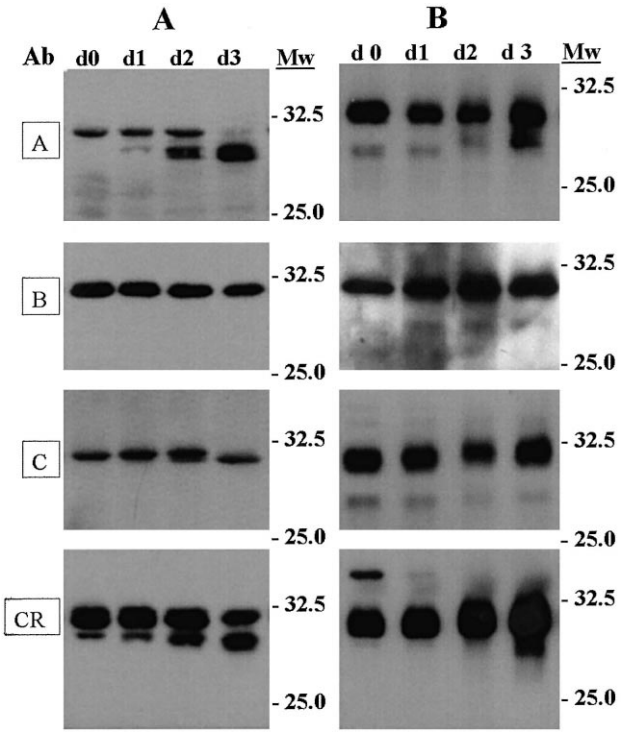

Fig. 2. Expression of 14-3-3 proteins in cytosol and microsomal fractions of barley embryos. Western analysis following SDS–PAGE, probed with specific antibodies (Ab) for the 14-3-3 isoforms (A: 14-3-3A, B: 14-3-3B and C: 14-3-3C) and a cross-reacting antibody (CR). The imbibition time of the barley grains in days is indicated with d0, d1, d2 and d3. Extracts of the dissected embryos were used for cell fractionation and samples (15 μg protein) were loaded. A: Cytosol fractions. B: Microsomal fractions.

To further examine these different forms of 14-3-3A in the cytosol and microsomal fraction, expression was studied in embryos during germination of intact grains. In the cytosol, the 30 kDa 14-3-3A band was found to decrease simultaneously with the increase of the 28 kDa band (Fig. 2A, upper panel). Moreover, the 28 kDa band appeared from day 1 on, which coincides with the moment of visible germination. For the 14-3-3B isoform in the cytosol (Fig. 2A, second panel), neither a change in molecular weight nor in protein level was observed. For 14-3-3C (Fig. 2A, third panel), the 31 kDa band decreased during germination, and a band with a slightly lower molecular weight appeared in the germinated embryos.

The examination of the three 14-3-3 isoforms in the microsomal fractions of the same germination series (Fig. 2B) revealed that all three isoforms were present at rather constant protein levels during germination. In contrast to what was found for 14-3-3A in the cytosol, the microsomal fraction contained a constant amount of the 30 kDa 14-3-3A and in addition, at day 2 and 3, also 28 kDa 14-3-3A protein was detected. For 14-3-3B and 14-3-3C, equal quantities of 31 kDa protein were found in all microsomal fractions, although 14-3-3C seemed to shift to a somewhat higher molecular weight. Western blots performed with the commercial cross-reacting anti-14-3-3 antibody (Santa Cruz), raised against a more conserved part of the 14-3-3 protein (amino acids 3–21), resulted

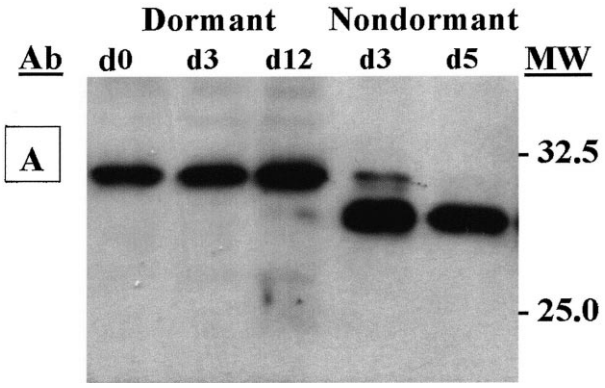

Fig. 3. Germination-related modification of 14-3-3A protein in the cytosol of barley embryos. Western analysis following SDS–PAGE, probed with specific antibody (Ab) 14-3-3A (A). Non-germinated embryos from dormant barley grains; dry (d0) and after imbibition in water at 20°C for 3 (d3) and 12 (d12) days. Germinated embryos from non-dormant barley grains were 3 (d3) and 5 (d5) days imbibed.

in several bands between 31 and 28 kDa in both the cytosolic and microsomal fractions (Fig. 2A,B, lower panels).

3.2. Germination-related decrease in molecular weight of 14-3-3A

The relation between the germination event and the appearance of the 14-3-3A 28 kDa form was further investigated with the following experiment. Dormant (non-germinating) and regular non-dormant grains were imbibed in water. While the non-dormant grains germinated after 1 day, the dormant grains did not germinate during the 12 days of imbibition. After imbibition, the dissected embryos were subjected to protein extraction and cell fractionation. In the cytosolic fraction of the germinated non-dormant embryos, the 28 kDa 14-3-3A was detected after 3 days (Fig. 3). No 28 kDa 14-3-3 could be discovered in the non-germinated dormant embryos, not even after up to 12 days of imbibition. Therefore, the appearance of the 28 kDa 14-3-3A form in the embryo seems to be related to germination.

No effect of Ca^{2+} or phosphatases was observed on the 30 or 28 kDa 14-3-3A (results not shown), suggesting that Ca^{2+} binding or phosphorylation do not play a role in the observed modification of 14-3-3A. To exclude the possibility that the observed modification of the 30 kDa 14-3-3A to a 28 kDa form occurs during the extraction procedure, a control experiment was done. For this control, germinated embryos were ground in liquid nitrogen and immediately transferred to 95°C SDS sample buffer. When the $10\,000 \times g$ supernatant of this sample was subjected to Western analysis, the 28 kDa band was found to be present as well (results not shown). In addition, no increase in the 28 kDa form was seen when extracts were stored for longer periods.

3.3. Effects of tryptic digestion on 14-3-3 proteins from barley embryos and heterologously expressed 14-3-3 proteins in E. coli

As proteolytic processing is a common phenomenon during germination of barley grains, we investigated the possibility that the 28 kDa 14-3-3A form was the result of proteolytic cleavage of the 30 kDa form. Since there are many putative trypsin digestion sites in the sequences of 14-3-3 proteins, we investigated if addition of trypsin to the 30 kDa 14-3-3A could produce a 28 kDa band. In an extract prepared from embryos of dry barley grains (day 0 in Fig. 4), the amount of 30 kDa 14-3-3A decreased upon incubation with trypsin and at the same time a 28 kDa 14-3-3A band appeared. This 28 kDa 14-3-3A band had exactly the same molecular weight as the one present in the cytosol of germinated embryos. A crude extract of germinated embryos containing 14-3-3A protein bands of 28 and 30 kDa (day 3 in Fig. 4) showed the decrease of both the 30 kDa and 28 kDa 14-3-3A bands upon trypsin digestion.

In the same experiment, tryptic digestion patterns of 14-3-3B and C did not show the formation of a 28 kDa product, but did show a decrease in the 31 kDa band. A slightly smaller band (approximately 30 kDa) was observed for 14-3-3B, but not for 14-3-3C. The cross-reacting antibody, which was raised against the conserved N-terminal part of the 14-3-3 proteins, recognized several bands between 31 and 28 kDa after tryptic digestion of either non-germinated or germinated embryo extracts.

Trypsin digestion profiles (Fig. 5A) showed that the digestion patterns for all three members of the 14-3-3 family, 14-3-3A, 14-3-3B and 14-3-3C, are very similar. All three sequences contain a comparable site positioned 10 amino acid residues from the C-terminal end of the protein (indicated with ▼ in Fig. 5A). This site corresponds with the most likely trypsin cleavage site in the *Arabidopsis* 14-3-3 protein GF14 omega [21]. Trypsin digestion of barley 14-3-3s could thus result in cleavage of the C-terminus. Since the epitope for anti-14-3-3B and C antibody recognition is at this position (amino acids 250–260), this would result in loss of antibody recognition (Fig. 5A). The specific antibody for 14-3-3A, however, was

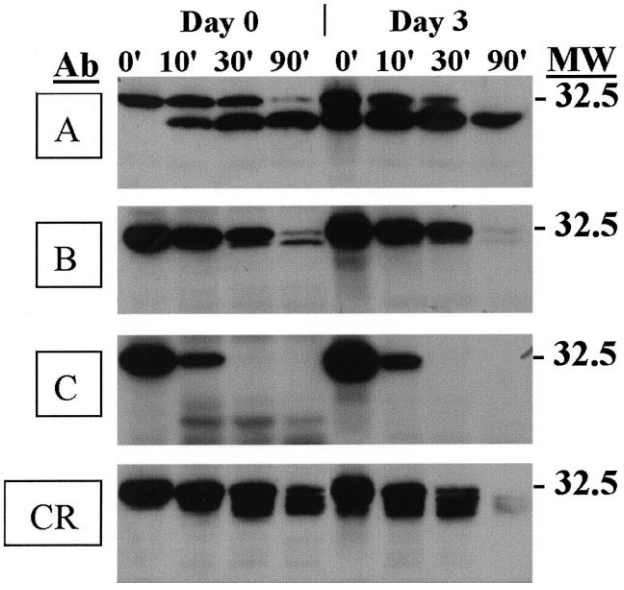

Fig. 4. Partial trypsin digestion of crude extracts from dry and germinated barley embryos. Western analysis following SDS–PAGE, probed with specific antibodies (Ab) for the 14-3-3 isoforms (A: 14-3-3A, B: 14-3-3B and C: 14-3-3C) and a cross-reacting antibody (CR). Crude extracts from dissected embryos from dry grains (day 0) and from 3 days imbibed, germinated grains (day 3) were prepared. Partial trypsin digestion was performed at 37°C with an enzyme to protein ratio of 1:100 for 0, 10, 30 and 90 min. Samples (15 μg protein) were loaded on gel.

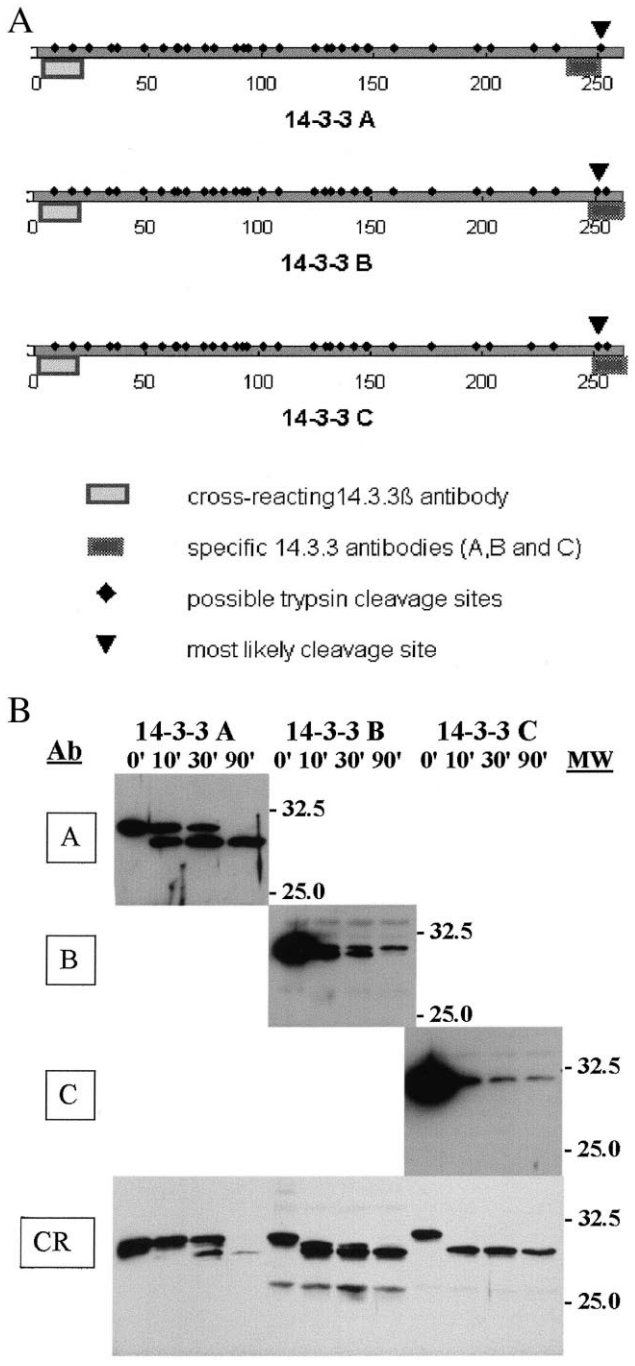

Fig. 5. Trypsin digestion of barley 14-3-3 proteins expressed in *E. coli*. A: Digestion profile and antibody recognition and B: Western analysis. A: Schematic digestion pattern for trypsin on 14-3-3A, 14-3-3B and 14-3-3C isoforms of barley and antibody recognition sites of the isoform-specific antibodies against barley 14-3-3A, B and C and the cross-reacting commercial 14-3-3β antibody. B: Western analysis following SDS–PAGE, probed with specific antibodies (Ab) for the 14-3-3 isoforms (A: 14-3-3A, B: 14-3-3B and C: 14-3-3C) and a cross-reacting antibody (CR). *E. coli* extracts expressing barley 14-3-3 proteins A, B and C (expression in *E. coli* as described in [14]) were digested with trypsin at 37°C, enzyme to protein ratio 1:10, during 0, 10, 30 and 90 min. Samples (0.1–1 μg protein) were loaded on gel.
←

raised against a peptide identical to the amino acids of positions 240–250.

Therefore, partial trypsin digestion of the separate isoforms, 14-3-3A, 14-3-3B and 14-3-3C, expressed in *E. coli* was carried out (Fig. 5B). Detection with the isoform-specific antibodies yielded the same result as the barley extracts. Only for 14-3-3A could a 28 kDa form be detected. However, when the same blots were incubated with the cross-reacting antibody (14-3-3β, Santa Cruz), not only 14-3-3A, but also 14-3-3B and 14-3-3C showed a degradation product of 28 kDa. This proved that all three isoforms, 14-3-3A, 14-3-3B and 14-3-3C, could be degraded at the C-terminal end of the protein in the same way. Due to the recognition site of the antibodies, in barley embryo extracts, the resulting 28 kDa band can only be detected for 14-3-3A. The smaller form of 14-3-3C present in barley embryo cytosolic extracts (Fig. 2A) was not formed upon tryptic digestion, suggesting that it is due to a different kind of post-translational modification. In contrast, a slightly smaller band was detected upon trypsin treatment of 14-3-3B, both in barley extracts and in *E. coli* (Figs. 4 and 5B), which was not detected in vivo during germination.

4. Discussion

The expression of 14-3-3 protein isoforms A, B and C was studied in different cell fractions of barley embryos during germination. Specific antibodies were used that were shown to be specific for one of the barley 14-3-3s, and did not cross-react with either one of the other isoforms [14]. The presence of all three isoforms was established in the nuclear, the microsomal and the cytosolic fractions of dry embryos as well as germinated embryos (Fig. 1).

However, subcellular differences in post-translational modification of barley 14-3-3 isoforms were observed. In the cytosolic and microsomal fractions, the appearance of a major 28 kDa 14-3-3A band was observed at the onset of germination. In contrast, the nuclear fractions of both non-germinated and germinated embryos mainly contained the 30 kDa form of 14-3-3A, and only minor amounts of different lower molecular weight bands (Fig. 1). Furthermore, a slightly smaller form of 14-3-3C was observed only in the cytosol (Fig. 2A). So, like reported before for *Arabidopsis* cells [20], different ratios of 14-3-3 bands were observed in the separate cell fractions of barley embryos. In addition, using isoform-specific antibodies, we can show that these bands represent different post-translational modifications of the same 14-3-3 isoforms.

We proceeded with further analysis of the 28 kDa 14-3-3A form. The increase of the 28 kDa 14-3-3A in the cytosol during germination coincides with the decrease of 30 kDa 14-3-3A, which makes it plausible that this is the result of a conversion. A number of observations on this modification can be explained by the idea that the 28 kDa form is the result of proteolytic cleavage of the C-terminus of the protein. However, although trypsin digestion experiments suggest cleavage of the C-terminus, it cannot be excluded that either N-terminal cleavage or other post-translational modifications of 14-3-3A take place during germination. Experiments to confirm whether the 28 kDa 14-3-3A protein is indeed the result of cleavage of the C-terminus are currently performed.

The observed modification of 14-3-3A during germination could be the beginning of total degradation and turnover of the protein. The fact that the induction of mRNA of 14-3-3A,

14-3-3B and 14-3-3C upon imbibition (0–24 h), reported before [14], is not followed by an increase in protein levels could be an indication for increased turnover. However, the observation of a rather constant amount of 28 kDa 14-3-3A in embryos after prolonged germination and the presence of 28 kDa 14-3-3A in leaf and root tissue of older plants (data not shown) do not support this degradation theory.

In conclusion, we show that all three barley 14-3-3 isoforms are present in the nucleus, the cytosol and the microsomal fraction of embryo cells. Differences in amounts of the 14-3-3 protein bands were shown to be due to differential post-translational modification. Possibly, processed forms of 14-3-3 are needed upon germination, which would suggest a difference in function for the 30 kDa and 28 kDa 14-3-3A forms. Our next goals are now to verify exactly what post-translational modification of 14-3-3 proteins takes place during germination, and to investigate the function of this modification.

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