Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck

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Rac signalling to actin—a pathway that is thought to be mediated by the protein Scar/WAVE (WASP (Wiskott–Aldrich syndrome protein)-family verprolin homologous protein)—has a principal role in cell motility. In an analogous pathway, direct interaction of Cdc42 with the related protein N-WASP stimulates actin polymerization[1]. For the Rac–WAVE pathway, no such direct interaction has been identified. Here we report a mechanism by which Rac and the adapter protein Nck activate actin nucleation through WAVE1. WAVE1 exists in a heterotetrameric complex that includes orthologues of human PIR121 (p53-inducible messenger RNA with a relative molecular mass (M_r) of 140,000), Nap125 (NCK-associated protein with an M_r of 125,000) and HSPC300. Whereas recombinant WAVE1 is constitutively active, the WAVE1 complex is inactive. We therefore propose that Rac1 and Nck cause dissociation of the WAVE1 complex, which releases active WAVE1–HSPC300 and leads to actin nucleation.

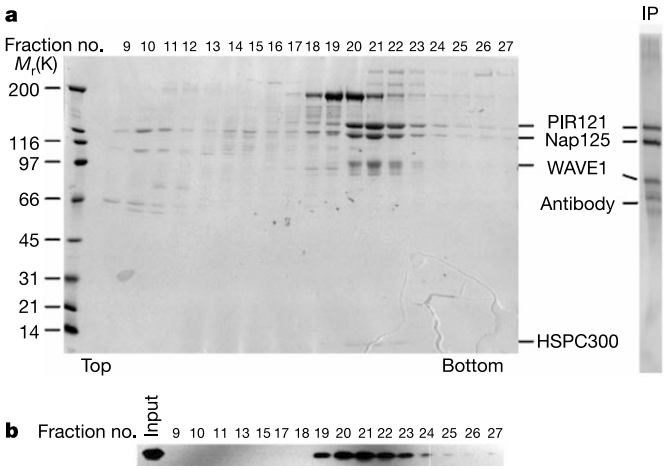

Figure 1 Purification of a WAVE1-containing complex from bovine brain. **a**, Left, Coomassie-stained 4–15% PAGE gel of fractions collected from a sucrose gradient. Right, fraction 21 was used for immunoaffinity purification (IP) using an antibody to WAVE1; the antibody heavy chain is indicated. The immunoaffinity-purified bands were analysed by MALDI. PIR121 was identified by 53 peptides covering 41% of AAD45723 (NCBI database accession number), Nap125 by 63 peptides covering 49% of NP_038464, WAVE1 by 22 peptides covering 38% of NP_003922 and HSPC300 by 12 peptides covering 45% of AAF28978. The results were confirmed by nanoelectrospray tandem mass spectrometry. HSP300 has been deposited in databases as a human gene fragment. The orthologous bovine and mouse sequences indicate a shorter coding sequence with a predicted M_r of 9K. **b**, Immunoblot using antibody to WAVE1 of the same sucrose gradient fractions as in **a**, showing an overlapping peak with the Coomassie-stained WAVE1 complex.

Members of the Rho family of small GTPases, such as Cdc42 and Rac1, and of the Src homology (SH) domain-containing SH2–SH3 adapter protein family, such as NCK, link extracellular signals and actin nucleation through pathways that include the WASP family of proteins and the actin nucleation machinery—the Arp2/3 complex[1]. All WASP family members contain a conserved verprolin-homology, cofilin-homology, acidic (VCA) domain that directly binds and activates the Arp2/3 complex. The Arp2/3 complex, in turn, catalyses the nucleation of actin filaments[2]. To prevent undesirable spontaneous actin nucleation in the absence of input signals, the activity of the WASP proteins is tightly regulated. For example, N-WASP is found predominantly in an autoinhibited conformation in which the carboxy-terminal VCA domain is occluded through interaction with the amino terminus of the protein[3,4]. When Cdc42 binds to the Cdc42/Rac1 interactive binding (CRIB) domain of N-WASP or when NCK binds to the polyproline region of N-WASP, this autoinhibition is relieved and the VCA domain is unmasked. Phosphatidylinositol(4,5)bisphosphate (PIP_2) can further activate N-WASP in cooperation with NCK or Cdc42 by binding to a basic region of N-WASP[5–7].

The WAVE proteins (WAVE1, WAVE2 and WAVE3 in mammals and orthologues in *Drosophila* and *Dictyostelium*[8–10]) are similar in structure to N-WASP[9]. They all have a C-terminal VCA domain, a polyproline region and a basic region. Unlike N-WASP, WAVE proteins do not contain a CRIB domain, and direct binding of WAVE1 to Rac1 has not been detected[8]. But much evidence suggests that WAVE1 functions downstream of Rac1. WAVE1 is translocated from the cytoplasm to membrane ruffles induced by Rac1 (ref. 8), and dominant-negative WAVE1 abolishes the formation of Rac1-dependent lamellipodia and Rac1-dependent neurite extensions[8].

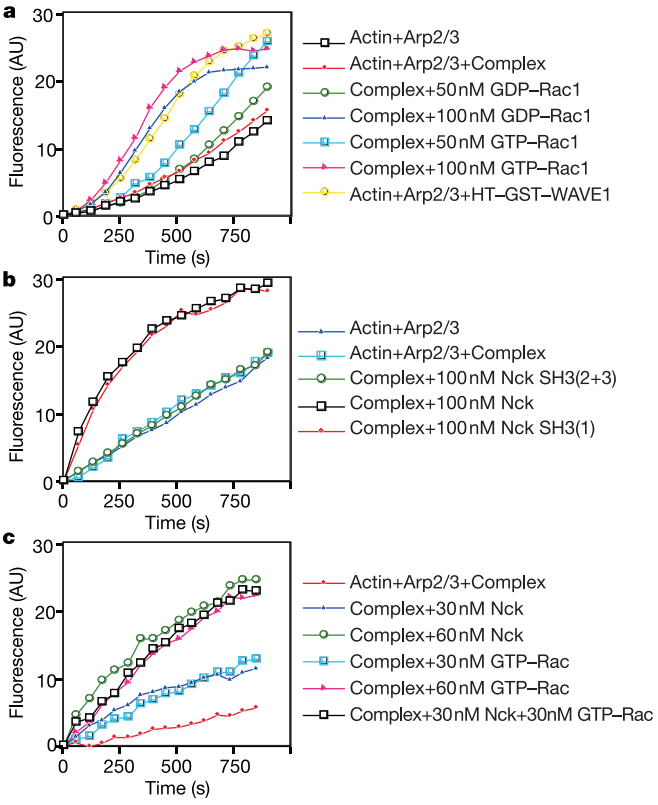

Figure 2 Rac1 or Nck induces Arp2/3 stimulation by the WAVE1 complex. Kinetics of Arp2/3-mediated actin polymerization in the absence or presence of the WAVE1 complex. GTP- or GDP-charged GST–Rac1 (**a**) or Nck fragments (**b**) or both (**c**) were added to reactions that included actin, Arp2/3 and the WAVE1 complex as indicated. Purified recombinant HT–GST–WAVE1 was added to actin and Arp2/3. AU, arbitary units.

The mechanism of regulation of WAVE1 is likely to be fundamentally different from that of N-WASP: whereas N-WASP is autoinhibited, recombinant WAVE1 is constitutively active in stimulating the actin nucleation activity of Arp2/3 (ref. 11). Therefore, WAVE1 activity is either inhibited in *trans* by other cellular regulators or regulated by post-translational modifications.

To identify potential *trans*-inhibitory proteins for WAVE1 activation, we searched for proteins that interact with WAVE1. We found that all of the detectable WAVE1 protein in soluble bovine brain extracts elutes in size-exclusion chromatography as a complex with an M_r of 500K (data not shown). We purified this complex to about 90% purity as judged by SDS polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie blue staining (Fig. 1a). Three proteins co-fractionated and were subsequently co-immunoprecipitated with WAVE1 (Fig. 1a).

The proteins were identified unambiguously by mass spectrometry as the bovine orthologues of the following human proteins: WAVE1; PIR121 (ref. 12), also named KIAA 0587 (ref. 13), pop (ref. 14) and CYFIP2 (ref. 15); Nap125 (ref. 16), also named NCKAP1 (ref. 17), KIAA0168 (ref. 18) and hem-2 (ref. 19); and HSPC300, which encodes a protein with an M_r of 9K (Fig. 1). The purified complex migrated with an *S* (Svedberg) value of 9.3 which, together with the calculated diffusion coefficient from the size-exclusion chromatography, predicts a theoretical M_r of 320K, consistent with a 1:1:1:1 stochiometry of the proteins in the complex. Both Nap125 and PIR121 have been identified as proteins that bind directly or indirectly to NCK, profilin II and GTP-charged Rac1. Neither WAVE1 nor HSPC300 has been observed previously in screens for Rac1-, NCK- and profilin-II-interacting proteins[14,16,20,21].

To test whether WAVE1 activity is inhibited in *trans* by the other proteins in the complex, we tested the ability of the complex to activate Arp2/3 using a pyrene-labelled actin polymerization assay. Whereas recombinant WAVE1 activated the Arp2/3 complex, the native WAVE1 complex did not (Fig. 2a). But the activity of the complex in Arp2/3 stimulation was increased markedly by GTPγS-charged Rac1 in a dose-dependent manner and to a lesser extent by GDP-charged Rac1. The half-time for polymerization was 3.5-fold shorter with GTPγS-Rac1 activation and 1.5-fold shorter with GDP-Rac1, which reflects both a shorter lag time for nucleation and an increased maximal elongation rate (Fig. 2a). By contrast, GTPγS-charged Cdc42 had no effect on the activity of the WAVE1 complex (data not shown). In the presence of GTPγS-charged Rac1, the WAVE1 complex attained a similar activity to that of an equal concentration of purified glutathione *S*-transferase (GST)–WAVE1 (Fig. 2a), which suggests that Rac1 is fully effective in relieving the inhibition of WAVE1 in the complex.

Because both Nap125 and PIR121 associate with NCK[16], we tested whether NCK can activate the WAVE1 complex. Both recombinant full-length NCK (Fig. 2b) and a recombinant fragment of NCK containing only the three SH3 domains of NCK (named SH3(1 + 2 + 3); data not shown) activated the WAVE1 complex, which shows that the SH2 domain of NCK is dispensable for activation. The association of Nap125 to NCK has been mapped to the first SH3 domain of NCK[16]. In agreement with this observation, the recombinant SH3(1) domain of NCK[5] activated the complex just as effectively as did full-length NCK (Fig. 2b). By contrast, an NCK protein fragment that only contained the second and third SH3 domains[5], SH3(2 + 3), had no activity (Fig. 2b), which indicates that SH3(1) is both necessary and sufficient for WAVE1 activation.

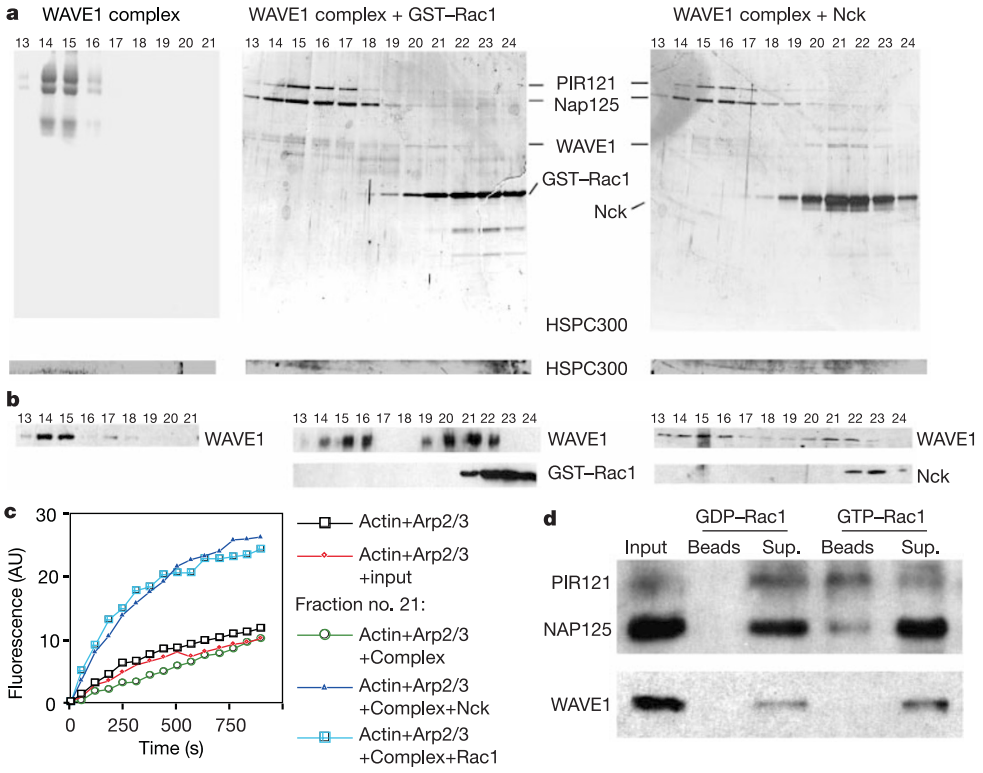

Figure 3 Disassembly of the WAVE1 complex by Rac1 and Nck enables activation. **a**, **b**, Purified WAVE1 complex was applied to a Superose 6 size-exclusion column in XB buffer with 10% glycerol alone (left) or in the presence of GTPγS-charged GST–Rac1 (middle) or Nck (right). Silver-stained gels of 4–20% PAGE (**a**) and immunoblots for WAVE1 and GST–Rac1 or Nck of 10% PAGE (**b**) are shown for each sample. The background bands in the right panel of **a** are present in the original Nck bacterial preparation. The lower panels in **a** are higher contrast exposures of the low M_r regions of the same gels. **c**, Actin polymerization assay of fraction 21 from the Superose 6 size-exclusion column. **d**, Immunoblots for PIR121, Nap125 and WAVE1 after co-precipitation assays with GST–GTPγS-Rac1 or GST–GDP-Rac1 beads. Shown are 10% of the input and the supernatant and 100% of the beads.

Neither NCK nor GTPγS-charged Rac1 further activated recombinant GST–WAVE1 (data not shown), which supports the idea that these activators function by relieving inhibition rather than by stimulating WAVE1 activity. Although N-WASP contains a basic region that mediates PIP_2-induced activation of N-WASP[5,6], the small basic region in WAVE1 does not seem to have a similar function because PIP_2 did not activate the WAVE1 complex further (data not shown). GTPγS-charged Rac1 and NCK activated the WAVE1 complex in an additive manner (Fig. 2c).

In a simple mechanism, Rac1 and NCK could relieve the inhibition of WAVE1 in the complex by stimulating disassembly of the complex and thereby releasing active WAVE1. To test this model, we analysed the purified complex in the presence or absence of the activator proteins by size-exclusion chromatography. Whereas the intact, inactive complex eluted at an M_r of 500K, on addition of NCK or GTPγS-charged GST–Rac1, the complex disassembled into two subcomplexes: WAVE1 and HSPC300, which co-fractionated as a complex of 180K; and PIR121 and Nap125, which co-eluted as a complex of 450K (Fig. 3a, b). Nap125 and PIR121 remained associated and could be co-immunoprecipitated (data not shown). The activator proteins (NCK and GST–Rac1) did not co-elute with PIR121 and Nap125, which possibly reflects a weakly bound complex that dissociated on passage through the column.

To determine whether disassembly of the complex contributes to activation of WAVE1, we tested the size-exclusion fractions with either NCK or GST–Rac1 for Arp2/3-dependent actin nucleation. The fractions containing PIR121 and Nap125 showed no activity (data not shown), whereas the WAVE1- and HSPC300-containing fractions (fraction 21) stimulated actin nucleation (Fig. 3c). These results indicate that NCK or Rac1 may cause disassembly of the complex and that this dissociation may activate WAVE1. To clarify further which of the proteins in the WAVE1 complex might associate with Rac1, we incubated beads coated with GTPγS-charged GST–Rac1 with bovine brain extract. Nap125 and PIR121, but not WAVE1, associated with the beads, but showed a much lower affinity for the GDP-charged GST–Rac1 that was used as a control (Fig. 3d).

In summary, although WAVE1 had been implicated as the downstream target of Rac1, no regulatory linkage had been found previously. Consequently, the important Rac1-dependent pathway for actin nucleation has not been described. Similarly, although the association of NCK and Rac1 with NAP125 and PIR121 has been observed in several screens[14,16,20,21], their role as regulators of actin nucleation has not been shown. Our results indicate that WAVE1, like N-WASP and WASP, mediates signals from NCK and the Rho GTPases. The activation mechanisms of WAVE1 and N-WASP are very different: N-WASP is autoinhibited, whereas WAVE1 is *trans*-inhibited. The action of Rac1 and NCK is to disassemble the *trans*-inhibited WAVE1 complex, which releases the active WAVE1 protein in association with HSPC300. Consistent with this model, Rac1 and WAVE1 do not colocalize in the lamellipodium: WAVE1 is localized at the extreme edge of the lamellipodium, whereas Rac1 is distributed diffusely over the lamellipodium[22,23]. Rac1 might remain bound to the NAP125 and PIR121 dissociated from the WAVE1 complex, but this has not been tested *in vivo*. Preliminary observations indicate that there is a similar regulation by *trans*-inhibition of WAVE2 in HeLa cells (H. Ho, R.R. and M.W.K., unpublished data).

Although the predominant regulation of WAVE1 activity described here is relief of *trans*-inhibition, an additional positive regulation by proteins that bind WAVE1 directly in an activator-independent manner is also possible. For example, IRSp53 has been reported to bind WAVE2 directly and enhance activation of Arp2/3 by recombinant WAVE2 (ref. 24). Preliminary data show that HSPC300, which remains associated with WAVE1 after activation, may also have a stimulating function on actin polymerization. The activation and dissociation of the WAVE1 complex process releases a subcomplex of NAP125 and PIR121, and this subcomplex may be free to interact with other cellular components. In this way, a Rac1 or NCK signal might potentially coordinate several cellular processes—similar to pathways that are activated by the α- and the βγ-subunits in heterotrimeric G-protein signalling.

Note added in proof: We have recently detected a fifth protein in the complex that co-migrates with WAVE1 on SDS–PAGE. We have identified this protein by mass spectrometry as the bovine orthologue of the human Abi2 (Abl Interactor 2) (NCBI database accession number NP_005750) (S.E., H. Ho, H. Steen and S. P. Gygi, unpublished work). Abi2 is highly homologous to hNap1 binding protein, previously described as a protein interacting with Nap125. Indeed, Abi2 appears to remain associated with the Nap125- Pir121 sub-complex upon dissociation of the WAVE1 complex (see Fig. 3). □

Methods

Purification of a WAVE1-containing complex

Throughout the purification, the WAVE1 complex was followed by immunoblotting with an antibody to WAVE1. All chromatography media described here were purchased from Pharmacia. Four bovine brains were cleaned and homogenized in XB (20 mM HEPES, pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA). The homogenate was centrifuged for 30 min at 14,000*g*, followed by a 30-min centrifugation at 225,000*g*. The supernatant was brought to 0.8 M ammonium sulphate, centrifuged at 10,000*g* for 10 min, and then incubated in batch with Butyl Sepharose Fast Flow for 2 h at 4 °C. After separating the flow-through from the beads, the beads were washed with five volumes of 50 mM potassium phosphate, pH 7.3, 0.4 mM EDTA containing 0.8 M ammonium sulphate, and the bound proteins were eluted in the same buffer containing 0.4 M ammonium sulphate. We concentrated the eluate (1.8 g of total protein) by precipitation with 65% ammonium sulphate. Alternatively, the brain homogenate was brought to 65% ammonium sulphate and the butyl-Sepharose binding step was omitted. The precipitate was dissolved in the same buffer, dialysed into 20 mM potassium PIPES, pH 6.8, 25 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA and 1 mM dithiothreitol (DTT), loaded onto a 50-ml Resource S column and eluted with a 25–400 mM KCl gradient developed over 15 column volumes. The WAVE1 peak fractions were dialysed into the same buffer, loaded onto a Mono S column and eluted under the same conditions.

The WAVE1-containing fractions were pooled, concentrated by ultrafiltration in a 3000 pressure cell (Amicon), and loaded onto a 330-ml prep grade Superose 6 column equilibrated in 20 mM potassium-Tris, pH 8.0, 100 mM KCl, 1 mM DTT. The WAVE1 complex eluted with an apparent Stokes radius of 8 nm. The peak fractions were pooled, loaded onto a MonoQ column, and eluted with a 100–500 mM KCl gradient developed over 10 column volumes. We pooled the complex-containing fractions and loaded them onto a 5–20% (w/v) sucrose gradient poured in 20 mM HEPES, pH 7.5, 150 mM NaCl and 1 mM DTT. The gradients were centrifuged for 24 h at 178,000 $g_{average}$ in a SW41 rotor, and fractions were collected from the top using a gradient fractionator (ISCO). The fractions from the gradient containing the peak WAVE1 immunoreactivity were used for immunoaffinity purification. Affinity-purified antibody to WAVE1 or rabbit γ-globulin (2 mg) was crosslinked to protein A beads by dimethyl pimelimidate. The beads were incubated with the WAVE1-containing fraction for 3 h in 4 °C, washed with XB supplemented with 500 mM NaCl and eluted with 100 mM glycine, pH 2.0.

Protein identification by mass spectrometry

Protein bands separated by one-dimensional PAGE and visualized by Coomassie blue staining were excised and digested in-gel with trypsin as described[25]. High mass accuracy matrix-assisted laser-desportion ionization (MALDI) peptide mapping was carried out on Reflex III instrument (Bruker Daltonics). We carried out nanoelectrospray peptide sequencing[26] on a quadrupole time-of-flight mass spectrometer (QSTAR, PE-Sciex) using the MDS-Proteomics nanoelectrospray ion source (MDS-Proteomics). Mass spectra were searched in non-redundant protein sequence databases and expressed sequence tag databases using the PepSea search engine (MDS-Proteomics).

Protein co-precipitation

Bacterially expressed GST–Rac1 bound to glutathione beads (2 mg ml^{-1}) was charged with GDP or GTP-γS as described[27] and incubated for 1 h in 4 °C with bovine brain high-speed supernatant supplemented with 1% Triton X-100. We washed the reactions three times in XB plus 500 mM KCl and twice in XB. The bound proteins were then eluted with SDS loading buffer.

Protein purification

Recombinant human Rac1 and NCK SH3(1) were expressed in *Escherichia coli* as GST fusion proteins; cleaved NCK, NCK SH3(1 + 2 + 3), NCK SH3(2 + 3) and GST–NCK SH3(2 + 3) were a kind gift from B. Mayer. Rac1 proteins were charged with GDT, GTP or GTP-γS as described[27]. We purified human WAVE1 from SF9 cells as a His–GST tag fusion protein. Arp2/3 complex was purified from bovine brain extract on butyl-Sepharose and DEAE Sepharose, followed by affinity purification on GST–VCA agarose and elution with 100 mM $MgCl_2$ (ref. 28). GST–VCA protein was expressed in *E. coli* and bound to glutathione agarose beads.

Actin polymerization assay

We prepared actin from rabbit muscle as described[29]. Pyrene-labelled actin (15% final labelling) at 1 μM was added to 80 nM purified His-tag–GST–WAVE1 or WAVE1-containing complex, 30 nM purified Arp2/3 complex in 20 mM HEPES, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA and 0.5 mM ATP. Bacterially expressed GST–Rac1 or NCK was added to a final concentration of 30 mM, 50 mM or 100 mM. The fluorescence was measured for 15 min at 21 °C using a Carry Eclipse instrument (Varian).

Antibodies

All the antibodies were generated in rabbit and affinity-purified as described[30]. Antibody to WAVE1 was generated using amino acids 436–560 expressed in *E. coli* as an immunogen. Antibody to Nap125 was generated against the peptide sequence CHAVYKQSVTSSA. Antibody to PIR121 was generated against the peptide sequence CNEVFAILNKYMKSVETDSST. Antibody to Rac1 was from Transduction Laboratories and antibody to NCK was from Neo Markers.

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Competing interests statement

The authors declare that they have no competing financial interests.

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Modulation of an RNA-binding protein by abscisic-acid-activated protein kinase

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Protein kinases are involved in stress signalling in both plant and animal systems. The hormone abscisic acid mediates the responses of plants to stresses such as drought, salinity and cold. Abscisic-acid-activated protein kinase (AAPK)—found in guard cells, which control stomatal pores—has been shown to regulate plasma membrane ion channels[1]. Here we show that AAPK-interacting protein 1 (AKIP1), with sequence homology to heterogeneous nuclear RNA-binding protein A/B, is a substrate of AAPK. AAPK-dependent phosphorylation is required for the interaction of AKIP1 with messenger RNA that encodes dehydrin, a protein implicated in cell protection under stress conditions. AAPK and AKIP1 are present in the guard-cell nucleus, and *in vivo* treatment of such cells with abscisic acid enhances the partitioning of AKIP1 into subnuclear foci which are reminiscent of nuclear speckles. These results show that phosphorylation-regulated RNA target discrimination by heterogeneous nuclear RNA-binding proteins[2] may be a general phenomenon in eukaryotes, and implicate a plant hormone in the regulation of protein dynamics during rapid subnuclear reorganization.

Guard cells regulate the apertures of microscopic stomatal pores on the leaf epidermis through which plants take up CO_2 and lose O_2 and water vapour. Appropriate regulation of stomatal aperture in response to environmental conditions, achieved by osmotic swelling and shrinking of guard cells, is vital for plant productivity and drought resistance[3,4]. We previously used *de novo* sequencing by tandem mass spectrometry to obtain peptide sequence information that allowed us to clone the complementary DNA encoding AAPK from the fava bean, *Vicia faba*[1]. Expression of a dominant negative form of AAPK in guard cells prevented activation by abscisic acid (ABA) of anion channels and stomatal closure, implicating AAPK in

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