Hyperosmotic-induced Protein Kinase N 1 Activation in a Vesicular Compartment Is Dependent upon Rac1 and 3-Phosphoinositide-dependent Kinase 1*

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Protein kinase N 1 (PKN1), which in part resembles yeast protein kinase C, has been shown to be under the control of Rho GTPases and 3-phosphoinositide-dependent kinase 1 (PDK1). We show here that green fluorescent protein-tagged PKN1 has the ability to translocate in a reversible manner to a vesicular compartment following hyperosmotic stress. PKN1 kinase activity is not necessary for this translocation, and in fact the PKN inhibitor HA1077 is also shown to induce PKN1 vesicle accumulation. PKN1 translocation is dependent on Rac1 activation, although the GTPase binding HR1abc domain is not sufficient for this recruitment. The PKN1 kinase domain, however, localizes constitutively to this compartment, and we demonstrate that this behavior is selective for PKNs. Associated with vesicle recruitment, PKN1 is shown to undergo activation loop phosphorylation and activation. It is established that this activation pathway involves PDK1, which is shown to be recruited to this PKN1-positive compartment upon hyperosmotic stress. Taken together, our findings present a pathway for the selective hyperosmotic-induced Rac1-dependent PKN1 translocation and PDK1-dependent activation.

Hyperosmotic stress is established as a potent activator of several signaling cascades, including stress-activated protein kinase 1 (1), p38 (2), and extracellular signal-regulated kinases (3). In contrast, protein kinase B (PKB) is shown to be down-regulated by hyperosmotic stress via dephosphorylation of its regulatory Thr-308 and Ser-473 phosphorylation sites (4). Interestingly, the yeast PKC homologues, which in part resemble the PKNs, have been established as essential for the maintenance of cell wall integrity (5). The regulation of cell wall integrity has been likened to the hyperosmotic stress response (6). The amino-terminal domain of the yeast PKC-related proteins (11). PKNs are activated by fatty acids and phospholipids in vitro, although the in vivo significance of this remains unclear (12, 13). The amino-terminal HR1 domain was identified as a Rho interacting region (14–16), and RhoB has been shown to target PKN1 to an endosomal compartment where it is implicated in controlling the kinetics of epidermal growth factor receptor traffic (17, 18).

The interaction of Rho with PKN1 has been demonstrated to facilitate PKN1 activation loop phosphorylation by 3-phosphoinositide-dependent kinase 1 (PDK1). PDK1 was originally purified as an activity responsible for PKBα activation loop phosphorylation (19, 20). PDK1 has been demonstrated more recently to phosphorylate equivalent residues on many other AGC kinases, including p70S6K, cAMP-dependent protein kinase, and PKCs (reviewed in Ref. 21). Based upon co-transfection experiments, the in vivo ternary complex of Rho-PKN1-PDK1 has been shown to be dependent on PI 3-kinase activity and to be critical for the catalytic activation of PKN1 (22). PKN1 has been linked to stress-induced pathways because it has been implicated upstream of c-Jun transcription via p38Y (23), both are activated upon hyperosmotic stress. Another relevant PKN response involves Fyn tyrosine kinase, which has been shown to mediate PKN2 function in keratinocytes (24) and has recently been shown (25) to be essential in transcription from the osmotic response element.

Here we describe the acute translocation of GFP-PKN1 to vesicles in response to hyperosmotic stress. It is established that PKN1 translocation is dependent on Rac1 activation, and the kinase domain of PKN1 is shown to be an essential component of this response. The activity of PKN1 is found not to be required for vesicle recruitment, although activation loop phospho-

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The abbreviations used are: PKB, protein kinase B; PKN, protein kinase N; PI, phosphatidylinositol; PKC, protein kinase C; GFF, green fluorescent protein; HA, hemagglutinin; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid; MBP, myelin basic protein; PAK, p21-activated kinase; GST, glutathione S-transferase; PDK1, 3-phosphoinositide-dependent kinase 1; GTPyS, guanosine 5′-3-O-(thio)triphosphate.
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phorylation and catalytic activation of PKN1 is shown to increase upon hyperosmotic stress. This activation is catalyzed by PKD1, which is recruited to the PKN1 compartment in a PI 3-kinase- and PKN1-dependent manner. Thus a pathway is established that leads from the plasma membrane activation of Rac1 to the vesicular accumulation of an activated PKN1 complex.

EXPERIMENTAL PROCEDURES

Materials—Anti-PKN1 monoclonal antibodies were from Transduction Laboratories, and the anti-phospho-PKN polyclonal antibody was as described previously (22). For immunofluorescence, the anti-Myc 9E10 monoclonal antibody was from Cancer Research UK monoclonal services. Cy3 anti-mouse antibody was from Jackson ImmunoResearch, and anti-HA polyclonal antibody was from Santa Cruz Biotechnology. The Rac1 activation assay was purchased as a kit from Totam Biologicals. Anti-GFP polyclonal antibody used for immunoprecipitation was purchased from Clontech, and phosphatidylinserine lipids were from lipid products. The PI 3-kinase inhibitor LY294002 and the ROCK/PKN inhibitor HA1077 were purchased from Calbiochem.

Plasmid Constructs—GFP-PKN1 and GFP-PKN2 constructs were generated by PCR using as templates PKN1 and PKN2 constructs, respectively. PKN1 was amplified using primer 1, 5' -GCAGCAAGTTC- GCATGGCAGCCGAGCAGTGATGCAGGC-3'; primer 2, 5' -GC- GCCTGATACCTGAAGACCCGACGCAATGCAAGTCGAGAG-3'; for PKN2 primer 3, 5' -GGCCAAAGTGATGTCTGCTGCCCACG-3'; and primer 4, 5' -GGCCCGGTACCTTAACACCAATC-3'. Primers 1 and 3 incorporated a HindIII restriction site, and primers 2 and 4 contained a KpnI site. The resulting products contained the full-length PKN sequences and were cloned into PCR blunt (Invitrogen). This was subsequently digested with HindIII and KpnI and cloned into pEGFP-C1 (Clontech) enabling the fusion of an amino-terminal GFP tag in-frame with the full-length PKN1 and PKN2. The DS-Red PKN1 kinase domain was derived from the full-length cDNA. The Myc-tagged PKN1 kinase domain was generated by cloning the kinase-dead K644R mutation from the kinase-dead kinase domain into the EcoRI/HindIII sites of the GFP-PKN construct. GFP-PDK1 was a gift from Dr. Dario Alessi, Dundee, UK, and the Myc-tagged Rac1 wild type and N17 plasmids were gifts from Dr. Alan Parkinson, unpublished observations. 3

Hyperosmotic Stress—Cells were subjected to hyperosmotic conditions for 30 min by the addition of Dulbecco's modified Eagle's medium containing 0.4 M sucrose and 25 mM Hepes, pH 7.2. For recovery experiments, cells were shocked for 30 min, and then medium was replaced with normosmotic medium. The average number of vesicles after 30 min of hyperosmotic stress and subsequent 15 and 30 min of recovery was determined. From each experiment, 10 GFP-PKN1 vesicle-containing cells were sampled. Images were taken as confocal sections through the center of the cell, and the number of vesicles from each image was counted.

Microscopy—Cells were seeded on acid-washed glass coverslips, and transfection and stimulation were as indicated in the figure legends. Notably, cells treated with hyperosmotic media for 30 min displayed a dramatic change in localization, with GFP-PKN1 expressed in NIH-3T3 cells displayed a punctate pattern. For each condition a 15-cm 2 plate of cells were transfected with GFP-PKN1 and stimulated with hyperosmotic medium as indicated in the figure legends. Cells were harvested in 400 μl of sample buffer and fractionated on a 10% SDS-polyacrylamide gel. Activation loop Thr-774 phosphorylation of PKN1 was assessed using a phospho-specific polyclonal antibody with excess of dephospho-epitope as described (22). Relative PKN1 phosphorylation was determined as a function of PKN1 protein levels defined using the PKN1 monoclonal antibody. GFP-PKN1 phosphorylation is taken from three duplicate experiments, where represented graphically the error bars denote the S.E.

RESULTS

GFP-PKN1 expressed in NIH-3T3 cells displayed a punctate cytoplasmic localization with an absence of nuclear localization. The effect of a range of potential PKN1 agonists on the subcellular distribution of GFP-PKN1 was investigated including the following: epidermal growth factor, fibroblast growth factor, insulin, platelet-derived growth factor, and stress conditions such as oxidative, temperature, hypo- and hyperosmotic stress. Notably, cells treated with hyperosmotic media for 30 min displayed a dramatic change in localization, with GFP-PKN1 accumulating in large cytoplasmic vesicular structures (Fig. 1A).

The dynamic nature of the hyperosmotic-induced structures was investigated by placing cells back into osmotically balanced media following 30 min of hyperosmotic stress. Cells containing GFP-PKN1 vesicles were randomly selected, and

3 S. Parkinson, unpublished observations. 4 H. Mellor, unpublished observations.
sectional images taken by confocal microscopy. These vesicles were clearly dissipated on removal from hyperosmotic conditions; quantitation showed that the average number of vesicles per cell decreased in a time-dependent fashion after re-addition of osmotically balanced medium. It is evident therefore that the accumulation of vesicular PKN1 is a reversible process (Fig. 1B).

GFP-PKCε has been shown to accumulate in vesicular structures following chronic PKC inhibition in MEF cells (26). To investigate the selectivity of the PKN1 translocation in response to hyperosmotic stress, DS-Red-PKN1 and GFP-PKCε were co-expressed. DS-Red-PKN1 has been co-expressed with GFP-PKN1 to confirm that they show the same localization behavior before and after hyperosmotic stress (data not shown). The ability of DS-Red PKN1 to translocate into vesicles upon hyperosmotic stress was unaffected by the presence of GFP-PKCε; furthermore, PKCε did not locate to the DS-Red PKN1-containing structures (Fig. 1C), indicating that the observed behavior of PKN1 in response to hyperosmotic stress shows some selectivity. The ability of other PKN subfamily members to respond to hyperosmotic stress was also followed. GFP-PKN2 when unstimulated was largely cytoplasmic with some limited nuclear accumulation (Fig. 1D). After hyperosmotic stress, GFP-PKN2 showed the same translocation pattern as GFP-PKN1 (Fig. 1D), indicating that the described behavior is a common PKN response.

Fig. 1. Reversible translocation of GFP-PKN1 in response to hyperosmotic shock. A, NIH-3T3 cells were transiently transfected with GFP-PKN1, and cells were either unstimulated (Control) or subjected to 30 min of hyperosmotic stress (Hyper) prior to fixation. B, the reversibility of the response in A was recorded by monitoring cells after 30 min of hyperosmotic stress and also following 15 or 30 min of recovery in normal osmotic medium post-stress treatment. GFP-PKN1 vesicle containing cells were selected randomly for each time point, and the average number of vesicles per cell section is indicated graphically. Error bars indicate the S.D. given by 10 cells from each time point. Representative cell sections from each time point are shown as an inset. C, NIH-3T3 cells were co-transfected with DS-Red-PKN1 and GFP-PKCε and stimulated as in A. D, NIH-3T3 cells were transfected with GFP-PKN2 and stimulated as in A. All images are representative of a single 1.0-μm Z optical section, and the scale bar is equivalent to 10 μm.
PKN has been shown to bind to and become activated by members of the Rho family of small GTPases via the regulatory HR1 domain and indeed has been shown previously to be recruited to an endosomal compartment by RhoB (17). We tested the involvement of Rho proteins in the PKN response to hyperosmotic stress by employing the C3 toxin from *Clostridium botulinum*. C3 toxin has been shown to inhibit specifically the Rho subfamily over other Rho family GTPases such as Rac1 (29). NIH-3T3 cells were transfected with GFP-PKN1 and then pre-loaded with C3-GST for 6 h at 5 μg/ml followed by subject- 

tion to hyperosmotic stress. The effectiveness of C3-GST cell loading was measured by an *in vitro* ribosylation assay and also by monitoring the extent of stress fiber disruption assessed by phalloidin staining (data not shown). The accumulation of PKN1-containing vesicles was independent of C3-GST treatment, implying that Rho function is not required for this re- 


diagram

**Fig. 2.** PKN1 activity is not required for vesicle recruitment. A, NIH-3T3 cells were transiently transfected with GFP-PKN1 and treated with 20 μM HA1077 for 1 h (Control) or pretreated with 20 μM HA1077 for 30 min followed by 30 min hyperosmotic stress maintained with 20 μM HA1077 (Hyper) as indicated. Cells were also transfected with the inactive mutant GFP-PKN1-KR and were either unstimulated (Control) or subject to 30 min of hyperosmotic stress (Hyper) as indicated prior to fixation. B, NIH-3T3 cells were co-transfected with GFP- 

The influence of catalytic activity on this process was inves- 

tigated by employing the drug HA1077, which has been shown to inhibit both PKN1 and PKN2 (27, 28). Pretreatment of 

GFP-PKN1-transfected cells with HA1077 followed by hyperosmotic stress did not prevent PKN1 vesicle recruitment. In- 

terestingly cells treated with HA1077 alone displayed an accumu- 

lation of vesicular GFP-PKN1 (Fig. 2A). To investigate fur- 

ther the contribution of PKN1 activity, we performed local- 

ization studies using the kinase-dead GFP-PKN1-K644R mu- 

tant. When unstimulated this inactive PKN1 mutant shows a 

punctate cytoplasmic distribution, and after hypotonic stress 

accumulates in vesicles (Fig. 2A) as observed for wild type 

gFP-PKN1. DS-Red-PKN1 and GFP-PKN1-KR were co- 

expressed to investigate whether either form of PKN1 could 

behave in a dominant fashion with respect to vesicle recruit- 

ment. Both wild type and kinase-dead PKN1 were cytoplasmic 

in unstimulated cells and co-localized in vesicles upon hypo- 

tonic stress (Fig. 2B). These findings imply that the kinase 

activity of PKN1 is not necessary for vesicle recruitment. How- 

ever, given the effect of HA1077 in unstimulated cells, PKN1 

could play a role in the exit from this vesicular compartment, 

which itself may be part of a constitutive endocytic pathway 

(see below and “Discussion”).
fected into NIH-3T3 cells. Under control conditions, myc-PKN1 kinase domain already displayed a partially vesicular distribution, and upon hyperosmotic stress, this vesicular localization became more pronounced (Fig. 4A). To test whether the kinase domain localizes to the same structures as the full-length protein, the Myc kinase domain was co-expressed with GFP-PKN1; after hyperosmotic stress they were found to co-localize in large vesicular structures (Fig. 4B). The observation that the PKN1 kinase domain can by itself localize to vesicles raises the possibility that the kinase domains of highly related PKC isoforms could behave in a similar manner. Myc-PKN1 kinase domain was co-expressed with the GFP-PKCa kinase domain. After hyperosmotic stress the kinase domain of PKN1 could be detected in vesicles; in these structures the kinase domain of PKCa was absent (Fig. 4C). These data indicate that vesicle targeting of PKN1 through the kinase domain is specific for this compartment and must follow distinct mechanisms when compared with a closely related kinase domain.

The HA1077 effects on the basal state and osmotic induced distribution of PKN1 (Fig. 2A) imply that its activity could be required for vesicle turnover. However, it is not clear whether activation of PKN takes place in response to osmotic stress. Activation loop phosphorylation is required for optimum catalytic activity of PKN1 (22). Phospho-specific polyclonal antibodies were used to assess the effect of hyperosmotic shock on GFP-PKN1 activation loop phosphorylation. A 2-fold increase in phos-
Phosphorylation after 30 min of hyperosmotic stress was detected on GFP-PKN1 (Fig. 5A). We were also able to detect an increase in endogenous PKN1 phosphorylation after hyperosmotic shock. Immunoprecipitated GFP-PKN1 from transiently transfected NIH-3T3 cells, either prior to or post-hyperosmotic shock, was used to determine directly the effect of osmotic stress on activity. After hyperosmotic shock, immunoprecipitated GFP-PKN1 displayed a specific activity 2-fold above un-shocked GFP-PKN1 (Fig. 5B). Additional duplicate reactions incorporated the PKN inhibitor 20 μM HA1077 in vitro. All reactions were performed in parallel as duplicates. Relative specific activity was determined as a function of immunoreactive GFP-PKN1 determined by Western blotting from the same filter used for detection of MBP phosphorylation. Band intensities were analyzed using NIH Image™, and error bars represent the S.E. (n = 3).

PDK1 has been shown previously to bind to and facilitate the activation loop phosphorylation of PKN1 (22). Given the finding that PKN1 is both phosphorylated and activated after osmotic stress, we assessed the potential involvement of PDK1 in the control of vesicular PKN1. GFP-PDK1 was co-expressed with DS-Red-PKN1. The localization of both proteins was cytoplasmic in unstimulated cells, but after hyperosmotic stress, GFP-PDK1 and DS-Red-PKN1 were found to be co-localized in vesicles (Fig. 6). PI 3-kinase influences PDK1 via its pleckstrin homology domain (32, 33) and the subsequent phosphorylation and activation of PKN1 (22). We examined the effect on osmotic responses following pretreatment with the PI 3-kinase inhibitor LY294002. After hyperosmotic stress, GFP-PDK1 was no longer recruited to DS-Red-PKN1-positive vesicles. Notably, DS-Red-PKN1 was still recruited to vesicles; however, these were smaller after the pretreatment with LY294002 (Fig. 6).
Discusssion

The results described here demonstrate that PKN1 is acutely regulated by hyperosmotic shock, through the assembly of a vesicular complex with the upstream kinase PDK1. This process is triggered by the activation of Rac1, although this alone is found to be insufficient for accumulation of vesicular PKN1. A distinct site of interaction between PKN1 and the described compartment appears to be required, and this is consistent with the finding that the kinase domain itself is selectively targeted to vesicles. Based upon use of an inactive PKN1 mutant, it is shown directly that PKN1 catalytic activity is not itself required for vesicle recruitment, a finding corroborated by the vesicular accumulation of PKN1 in the presence of the catalytic inhibitor HA1077. Once vesicular, the subsequent recruitment of PDK1 leads to the increased activation loop phosphorylation of PKN1 that parallels its catalytic activation.

Recent studies (30) have reported the GTP loading of Rac1 in response to hyperosmotic stress, a finding confirmed here in a distinct cell type. In fact a hyperosmotic stress response has been described previously for another Rac1 effector, p21-activated protein kinase \( \gamma \)-PAK, which binds to and is activated by Rac1. \( \gamma \)-PAK has been shown to translocate from a soluble to a particulate fraction and become activated in response to hyperosmolarity (34). Interestingly, it was demonstrated that the activation but not translocation of \( \gamma \)-PAK was sensitive to wortmannin, suggesting a two-step mechanism for the \( \gamma \)-PAK response to hyperosmotic stress. This parallels the situation described here for PKN1 where inhibition of PI 3-kinase does not prevent vesicle accumulation of PKN1, while blocking recruitment of the upstream kinase PDK1. It has been shown previously (22) that inhibition of PI 3-kinase will block activation loop phosphorylation of PKN1.

The nature of the induced PKN1 vesicular compartment described here is not resolved. Immunostaining indicates that this is not an early endosomal compartment (EEA1-negative) nor an acidified compartment (lysotracker negative; data not shown). However, the effect of PI 3-kinase inhibition on the induced PKN1-positive compartment would be consistent with this being part of an endocytic pathway, where inhibition of PI 3-kinase arrests homotypic fusion (35).

Based upon experiments with the ADP-ribosylation factor C3 toxin, it is concluded that Rho-GTP interaction is not required for the recruitment of PKN1 in response to hyperosmotic shock. However, because Rac1 has also been reported to bind the HR1abc domain of PKN1 and also because Rac1 becomes GTP-loaded in response to hyperosmotic shock, the possible role of this PKN effector was investigated. The ability of the dominant negative Rac1 to suppress PKN accumulation in the vesicular compartment indicates that Rac1 plays a key role in this hyperosmotic induced entry event. However, this role is permissive for subsequent events rather than sufficient, because the HR1abc domain of PKN1 is not recruited to vesicles despite retaining the Rac1 (and Rho) interacting domain (14–16). The finding that the PKN1 kinase domain is in part constitutively vesicular, coupled with the observation that full-length PKN1 requires Rac1 activation, suggests that Rac1 binding to PKN1 on hyperosmotic stress induces a conformational change exposing the catalytic domain and hence allowing vesicular recruitment. Consistent with this, it has been suggested that the interaction of Rho GTPases at the amino-terminal HR1 motif acts to disrupt an autoinhibitory intramolecular interaction thereby allowing activation, i.e., an open conformation (36).

The incubation of Rho-GTP with PKN1 has been shown to increase the phosphorylation and catalytic activity of PKN1 (14–16), and the behavior of PKN1 described here supports the view that the allosteric input through the amino-terminal HR1 domain is required for complex formation with and subsequent phosphorylation by PDK1. The recruitment of PDK1 occurs through the kinase domain (results not shown) and is likely to involve the FXFDY motif described as a PDK1-docking site (37). This vesicular interaction is not responsible however for the accumulation of PKN1, because this still occurs when PDK1 recruitment is blocked by the 3-kinase inhibitor LY294004. Thus the kinase domain docking in the vesicle membrane must be determined by a distinct protein (or perhaps lipid) contact. The inhibitory effect of LY294002 on PDK1 recruitment to PKN1 indicates that PI(3,4,5)P3 is required to influence the conformation of PKN1 or that of PDK1 to facilitate complex formation. Both of these proteins have been shown to be influenced by PI(3,4,5)P3; however, in the case of PKN1 no specificity was observed relative to the precursor lipid PI(4,5)P2 (13). Hence it is likely that the role of PI(3,4,5)P3 is to enhance membrane occupancy of PDK1 through its pleckstrin homology domain (32) and so facilitate recruitment to the membrane-bound PKN1. The reduction in size of PKN1-positive vesicles on treatment of shocked cells with LY294002 suggests that the larger vesicles observed are a consequence of a PI 3-kinase-dependent vesicle fusion event; this is a characteristic of homotypic endosomal fusion (35).

The combined Rac1/PI(3,4,5)P3 regulatory input that facilitates PDK1 phosphorylation of PKN1 provides further evidence for the view that the specificity of PDK1 actions is driven by the co-association of regulatory inputs to target kinases (21). In this context it is notable that despite the requirement of PI 3-kinase for PDK1 recruitment, PKB, which is also recruited by 3-phosphoinositides, is not recruited to this hyperosmotic induced endosomal compartment (data not shown). This suggests that PKB is either actively removed or that under appropriate activating conditions other stabilizing interactions are required for PDK1 phosphorylation of PKB.

Hyperosmolarity is a known apoptotic stimulus (38, 39), and PKNs have been shown to undergo caspase cleavage in re-
response to apoptotic stimuli and under ischemic conditions (40, 41). However, we have not observed cleavage of PKN1 under osmotic stress. The responses defined here indicate that in fact the behavior of PKN1 observed under hyperosmotic conditions reflects an underlying constitutive process. The partial vesicular localization of the kinase domain in the absence of hyperosmotic shock and the observation that HA1077 induces some PKN1 vesicle accumulation suggest that this is a constitutive trafficking pathway up-regulated by hyperosmotic shock. The findings indicate that the PKN1 response to hyperosmolarity is not simply targeting PKN1 for degradation but that PKN1 activity is involved in the turnover or exit from this vesicular compartment.

In summary we describe PKN1 to be a stress-responsive kinase, with the induced translocation being selective over related PKCs, although shared with PKN2. Many stress induced signaling cascades are generalized stress responses, although upon investigation of other stresses, such as temperature shock and oxidative stress, no such PKN1 translocation was observed (data not shown). We conclude that the described PKN1 response is specific for hyperosmotic stress. The selective translocation of PKN1 coupled with its subsequent activation details a mechanism by which hyperosmotic shock can elicit a particular repertoire of responses through this kinase.

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