

The IRAK Homolog Pelle Is the Functional Counterpart of I κ B Kinase in the *Drosophila* Toll Pathway

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Abstract

Toll receptors transduce signals that activate Rel-family transcription factors, such as NF- κ B, by directing proteolytic degradation of inhibitor proteins. In mammals, the I κ B Kinase (IKK) phosphorylates the inhibitor I κ B α . A β TrCP protein binds to phosphorylated I κ B α , triggering ubiquitination and proteasome mediated degradation. In *Drosophila*, Toll signaling directs Cactus degradation via a sequence motif that is highly similar to that in I κ B α , but without involvement of IKK. Here we show that Pelle, the homolog of a mammalian regulator of IKK, acts as a Cactus kinase. We further find that the fly β TrCP protein Slimb is required in cultured cells to mediate Cactus degradation. These findings enable us for the first time to trace an uninterrupted pathway from the cell surface to the nucleus for *Drosophila* Toll signaling.

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Introduction

Toll and Toll-like receptors (TLRs) govern the nuclear localization and activity of NF- κ B and other Rel-related transcription factors via an evolutionarily conserved signal transduction pathway [1,2,3]. Prior to Toll signaling, Rel proteins are held in the cytoplasm by a tightly bound inhibitor that belongs to the I κ B family. Activated Toll receptors relay signals that trigger I κ B protein degradation, freeing the Rel proteins to translocate into nuclei, bind DNA, and regulate gene expression.

In the fruit fly *Drosophila melanogaster*, Toll signaling functions in both development and immunity [4,5]. Toll first acts in the syncytial embryo, where spatially graded signaling establishes the dorsoventral axis. In larvae and adults, Toll mediates the humoral immune response to fungi and Gram (+) bacteria. Embryonic axis formation requires the Rel protein Dorsal, whereas innate immune responses involve either Dorsal or the Dorsal-related immune factor (Dif), another Rel protein [6]. Cactus, a fly I κ B protein, is the inhibitor for both Dif and Dorsal. The adaptor proteins MyD88 and Tube, as well as the protein kinase Pelle, transduce signals from Toll to Cactus. These three signal-relay proteins each contain a death domain, a protein-protein interaction motif that mediates formation of a submembranous Toll signaling complex [7,8].

Like *Drosophila* Toll, most mammalian TLRs signal via three death domain proteins. Indeed, mammalian MyD88, IRAK4 and IRAK1 are the counterparts of fly MyD88, Tube, and Pelle, respectively [9,10]. However, additional factors link the death domain complex to I κ B in mammalian innate immune signaling. In particular, signaling by the IRAK 1,2, and 4 proteins requires the adaptor TRAF6, the TAB proteins, the protein kinase TAK1, and the I κ B Kinase (IKK) complex [1,11]. IKK-mediated phosphorylation of I κ B α at two sites, Ser32 and Ser36, triggers

ubiquitination, leading to proteasome mediated I κ B α degradation [12,13].

The Toll responsive sites in I κ B α and Cactus share substantial sequence similarity [12,14,15]. In fact, the signal-responsive domain of I κ B α can functionally substitute for the corresponding region of the Cactus protein [16]. Surprisingly, the *Drosophila* IKK does not function in the fly Toll pathway [17,18,19].

The fact that Toll-directed Cactus phosphorylation is IKK independent leads to two important questions. First, why are the signal responsive sites conserved when the kinase is not? Second, what kinase phosphorylates these sites in response to Toll activation? Here we address these two questions.

Results

Drosophila β -TrCP can Mediate Cactus Degradation

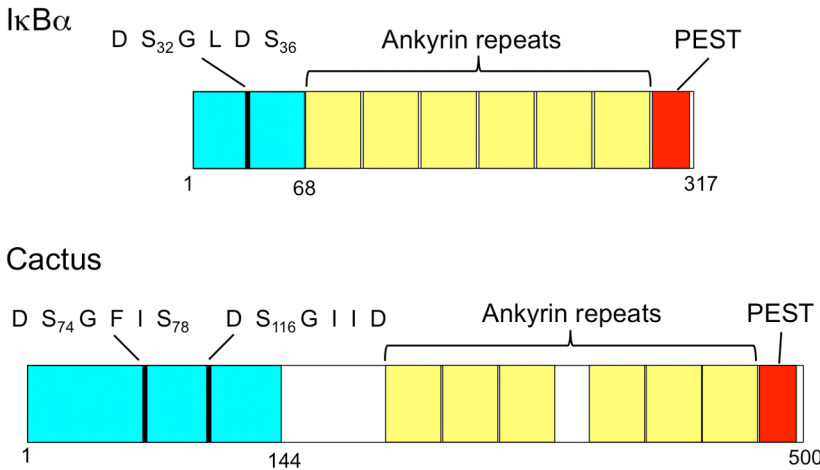
To explain the sequence similarity of the signal responsive sites in I κ B α and Cactus, it has been proposed that Cactus, like I κ B α , is targeted for degradation by a β -TrCP protein acting as the substrate recognition subunit for an SCF E3 ubiquitin ligase [16,20]. In mammals, the F-box/ β -TrCP protein of the SCF (Skp1/Cullin/F-Box) complex binds specifically to the phosphorylated form of the I κ B α motif DS₃₂GLDS₃₆. This recognition initiates ubiquitination, generating the signal for proteasomal recognition and proteolysis [1,21,22]. The recognition site for β -TrCP is thus a degron that initiates protein degradation in response to phosphorylation. Characterization of the degrons of a number of β -TrCP targets has demonstrated that they are highly similar in sequence, with a consensus sequence of DSGxxS, but are regulated by a diverse set of protein kinases (Figure 1A).

As shown in Figure 1B, both of the signal-responsive sites in Cactus exhibit similarity to β -TrCP sites, with the sequence from residues 73–78 forming a perfect match to the consensus

A.

Protein	β TrCP Degron	Kinase
I κ B- α	LLDDRHD SGLDS MKDEEY	IKK
β -Catenin	QQQSYLD SGIHS GATTTA	GSK3
VPU	I I ERAED SGNES DGDQQE	CKII
Bora	SSNIQM DSGYNT QNCGSN	Plk1
DEPTOR	SMSSCGS SGYFSS SPTLS	mTor

B.



C.

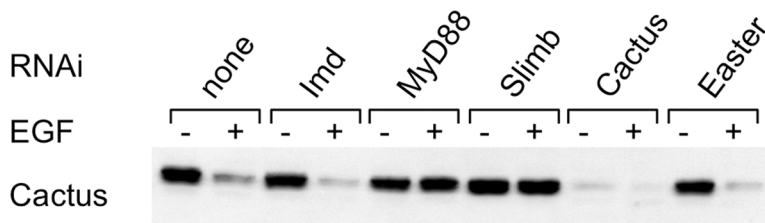


Figure 1. Role of Slimb β TrCP in Toll-directed Cactus degradation. A. The β TrCP recognition sites and protein kinases of well-characterized degrons (see [21], and references therein). B. Schematic drawing of I κ B and Cactus proteins (adapted from [16]). Teal colored regions represent signal-responsive domains. C. Anti-Cactus immunoblot of EGFR-Toll expressing S2 cells treated with indicated dsRNA's and activated with EGF. doi:10.1371/journal.pone.0075150.g001

recognition site. In flies, however, evidence for a role of β -TrCP protein in Cactus degradation is somewhat contradictory. The *slimb* locus is the sole β -TrCP gene in the *Drosophila* genome. Whereas a loss-of-function mutation in *slimb* blocks the Toll-dependent transcription of the patterning genes *snail* and *twist* in embryos, Toll-dependent transcription of the antifungal gene *drosomycin* is unaffected in adults [20,23]. Furthermore, ectopic expression of the viral β -TrCP inhibitor VPU in flies reduces but does not eliminate Pelle-mediated degradation of Cactus [20].

To investigate further the role of the Slimb β -TrCP in Cactus degradation, we used S2 cells transfected with a construct fusing the extracellular domain of EGFR to the transmembrane and intracellular domains of Toll. We have previously demonstrated that this chimeric receptor renders the Toll pathway responsive to EGF, directing Cactus degradation that is detectable within one minute of EGF addition [24]. To assay the requirement for Slimb

in Toll signaling, we used EGF activation of EGFR-Toll in combination with RNA interference (RNAi). The results were unequivocal. As shown in Figure 1C, RNAi against *slimb* abrogated Toll-directed degradation of Cactus. Indeed, eliminating *slimb* function had the same effect as did RNAi against *MyD88*, an essential component of the fly Toll pathway. In contrast, RNAi against *easter*, a gene that acts upstream of Toll, or against *Imd*, a gene essential to the IKK-dependent immune pathway, was without consequence. We conclude that Toll-directed Cactus degradation in these cells requires the β -TrCP protein Slimb.

Pelle Phosphorylates Signal-dependent Sites in Cactus

Given that IKK does not mediate Toll signaling, what kinase phosphorylates Cactus in response to Toll activation? A logical candidate is Pelle. The protein kinase activity of Pelle is strictly required for Toll signaling and forced expression of Pelle drives

Cactus degradation [15,25,26,27]. Furthermore, Pelle undergoes autophosphorylation, autoactivation, and colocalization with Tube, events that occur concomitant with signaling [28,29,30]. There is also the fact that forward genetic screens and genome-wide RNAi screens have failed to identify any other protein kinase required for Cactus degradation [31,32,33,34]. We therefore set out to assay Pelle as a Cactus kinase.

We began by expressing recombinant forms of Cactus and Pelle in *Escherichia coli*. We expressed full-length Cactus (500 aa), isolated the protein from inclusion bodies, and carried out solubilization and renaturation by the method of Nüsslein-Volhard and colleagues [35]. Expressing Pelle in *E. coli* proved more problematic, due to the frequent appearance of mutations that blocked Pelle production or inactivated the kinase domain. To circumvent the toxicity of an active Pelle kinase in bacteria, we adopted the strategy of co-expressing an antagonizing protein phosphatase [36,37,38]. For this purpose we chose λ protein phosphatase, which contains just 221 residues, is readily expressed in bacteria, and has very broad substrate specificity [39,40].

To ensure coordinated expression of Pelle and the λ phosphatase, we constructed a cistron consisting of a GST-Pelle fusion gene immediately 5' to the phosphatase gene. We induced expression in *E. coli* and used affinity chromatography to purify the Pelle fusion protein away from the λ phosphatase and other proteins. Upon incubation at high concentration with Mg²⁺ and ATP, recombinant Pelle protein underwent autoactivation and autophosphorylation, as evident in a slight decrease in electrophoretic mobility (Figure 2a). Pelle from *Drosophila* tissues similarly undergoes autoactivation and autophosphorylation following phosphatase treatment [30].

With an active Pelle preparation in hand, we set out to investigate Cactus as a potential substrate. We prepared two His₆-Cactus isoforms, the wild-type Cact^{wt}, and the triple mutant Cact^{S74A,S78A,S116A}. In the latter isoform, the serine-to-alanine substitutions at positions 74, 78, and 116 (see Figure 1B), are sufficient to block Toll-dependent degradation of Cactus *in vivo* [14,15,16]. We incubated Pelle with the Cactus proteins in the presence of [γ -³²P]-ATP and assayed phosphorylation by gel

electrophoresis and autoradiography. We observed robust phosphorylation of wild-type Cactus, but dramatically reduced phosphorylation of the triple mutant (Figure 2B). Indeed, although the residues altered in this mutant represent only 6% of the serines in Cactus, mutating these three sites consistently reduced phosphorylation by 75–80%.

Next, we compared the site specificity of Pelle with that of Casein Kinase II (CKII). We have previously demonstrated that CKII phosphorylates target residues in the carboxyl-terminal PEST domain, mediating Toll-independent destabilization of Cactus [41]. To compare the activity of the two kinases toward Cactus, we used as substrate Cact^{S74A,S78A,S116A}, in which the signal-dependent sites are mutated, and Cact^{ΔPEST}, in which the signal-independent sites are deleted. As shown in Figure 2C, Pelle and CKII exhibited reciprocal specificity. Under conditions where Pelle had only minor activity toward substrate Cact^{S74A,S78A,S116A}, CKII mediated extensive radiolabeling of this protein. Similarly, Pelle had robust activity toward Cact^{ΔPEST}, whereas CKII catalyzed phosphorylation of this substrate was virtually undetectable. We conclude that Pelle acts as a Cactus kinase and preferentially phosphorylates Cactus at the serines required for signal responsiveness.

Pelle Phosphorylates Signal-responsive Sites in I κ B α

Stein and colleagues have shown that *Drosophila* Toll signaling can target the signal dependent sites of I κ B α as well as Cactus [16]. We therefore predicted that Pelle should exhibit *in vitro* activity toward the two serines in the I κ B α degron, GS₃₂GLDS₃₆. To test this hypothesis we turned to a chimeric substrate, I κ B-Cact Δ 144, in which the signal-responsive region (residues 1–68) of I κ B α replaces the corresponding region (residues 1–144) of Cactus (see teal colored regions in Figure 1B). This chimera not only functionally substitutes for Cactus in fly embryos, but also strictly requires residues Ser32 and Ser36 for this activity [16].

We expressed and purified I κ B-Cact Δ 144 from bacteria. We then assayed Pelle activity using an anti-I κ B α serum that is specific for the I κ B α phosphorylated at both Ser32 and Ser36 in the degron. As shown in Figure 3, the phospho-specific antiserum detects a strong, Pelle-dependent signal, confirming that, as predicted, Pelle directly modifies the I κ B α degron, GS₃₂GLDS₃₆.

Discussion

Phosphoregulation of Cactus Stability

Studies in fly embryos have identified two processes, one signal independent and one signal dependent, that regulate Cactus stability [42]. The kinase responsible for each activity is now known. We previously used an in-gel kinase assay to purify the kinase mediating signal independent degradation [41]. We identified this kinase as Casein Kinase II (CKII) and showed that

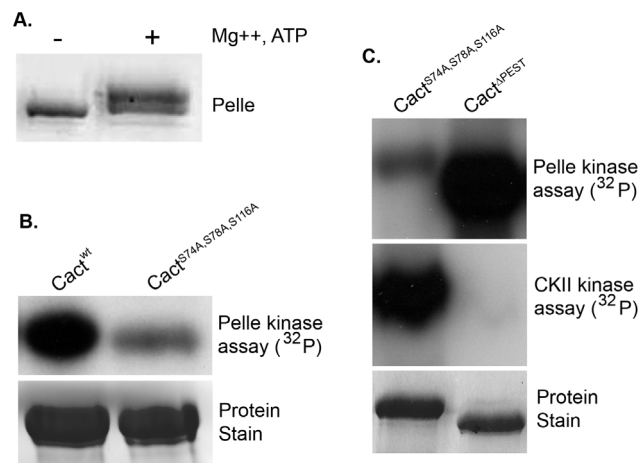


Figure 2. Pelle-mediated Cactus phosphorylation exhibits specificity for Toll-responsive sites. A. Autophosphorylation of recombinant GST-Pelle, stained with Coomassie brilliant blue. B. (Top) Autoradiogram of Cactus isoforms phosphorylated with GST-Pelle in the presence of [γ -³²P]-ATP. (Bottom) SDS-PAGE of unlabeled substrates. C. Autoradiograms of Cactus isoforms radiolabelled with GST-Pelle or Casein Kinase II (CKII) in the presence of [γ -³²P]-ATP. doi:10.1371/journal.pone.0075150.g002

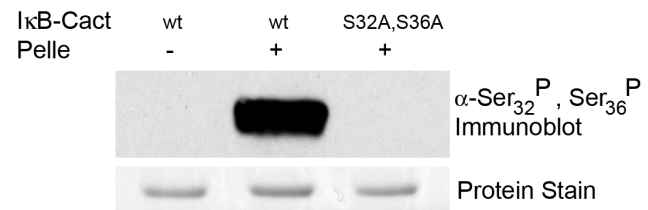


Figure 3. Pelle phosphorylates the I κ B α degron. Isoforms of the I κ B-Cact Δ 144 chimera were incubated with or without recombinant Pelle and the reaction products immunoblotted with an antiserum specific for the phosphorylated forms of serines 32 and 36. doi:10.1371/journal.pone.0075150.g003

it destabilized Cactus by modifying consensus target residues in the carboxyl-terminal PEST domain. Mammalian CKII similarly modifies and destabilizes $\text{I}\kappa\text{B}\alpha$ [43]. Here, we have shown that Pelle phosphorylates the signal-regulated sites in the amino-terminal domain of Cactus. Work from our lab and others has also shown that Pelle protein kinase activity is required downstream of Tube to direct Cactus degradation in response to Toll activation. Pelle thus has the *in vivo* and *in vitro* properties expected of the Cactus kinase.

Functionally, Pelle-catalyzed phosphorylation of Cactus is analogous to that of the mammalian IKK complex acting on $\text{I}\kappa\text{B}\alpha$. Different kinases mediate phosphorylation, but at highly similar sites. This site conservation has been suggested to reflect a need for SCF mediated recognition, a hypothesis supported by our finding that the Slimb β -TrCP is required in S2 cells for Cactus degradation. We note, however, that the Slimb β -TrCP may not mediate Cactus degradation in all cells and at all stages, since Lemaître and colleagues have provided evidence that Slimb is not the sole mediator of Cactus degradation in adult flies [20]. Involvement of an additional F box protein could explain why the Cactus of flies and other invertebrates typically contains two degrons, with the more carboxyl-terminal motif deviating slightly but consistently from the β -TrCP consensus (Figure 1 and unpublished data).

Pelle as the *Drosophila* Toll Pathway Kinase

The kinase activity of Pelle appears to fulfill multiple roles in Toll signaling. We have shown here that Pelle modifies Cactus. In addition, we and others have shown previously that Pelle phosphorylates Tube *in vitro* [27,28]. This activity may play an *in vivo* role in terminating signal transduction, since Pelle activity as a protein kinase mediates feedback regulation on Tube localization [27]. In addition, it is known that Dorsal undergoes signal dependent phosphorylation and can respond to Toll signaling in the absence of Cactus [44,45,46,47]. We speculate that Pelle is also responsible for modification of Dorsal *in vivo*.

By themselves, Pelle and Cactus do not appear to stably interact [28,48]. However, Pelle binds to Tube; Tube and Pelle both bind to Dorsal; and Dorsal binds to Cactus [28,45,46,48,49,50,51]. Taking all of these interactions into consideration, we can now generate an illustration of the overall pathway in *Drosophila* embryos for signaling by activated Toll receptors (Figure 4).

The protein-protein interactions shown in Figure 4 likely increase the efficiency or specificity of Pelle activity when Dorsal is the Toll target. They may, for example, provide steric constraints on the interaction of Pelle and Cactus that enhance site preference. They might also explain why a Cactus isoform lacking the destabilizing effect of the PEST domain requires the presence of Dorsal for efficient response to Toll signaling [16]. However, the same interactions may not be important when Dif is the target, since Tube fails to bind Dif under conditions in which it stably binds to Dorsal [9]. Why this difference? One possibility is that the establishment of a nuclear concentration gradient of Dorsal across the embryo requires much more tightly regulated protein localization and diffusion than does the nuclear import of Dif in response to immune challenge.

Although recruitment of Toll components into a signaling complex likely influences Pelle activity, several lines of evidence reveal that such a complex is not strictly required for the action of Pelle on Cactus. First, deleting the region of Tube that mediates binding to Dorsal has a relatively mild effect on Toll signaling [48,50,52]. Second, expression of a fusion construct that targets Pelle to the plasma membrane can effect signaling in the absence of Tube [28,53]. Third, activation of Toll in embryos by injection

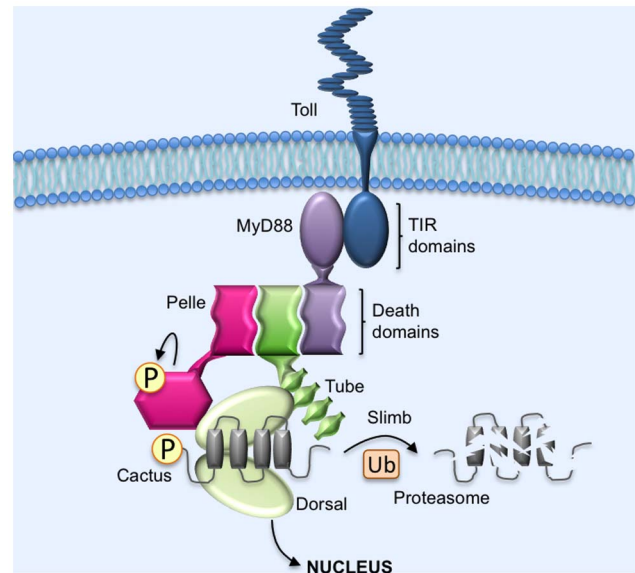


Figure 4. Model for Toll signaling in *Drosophila* embryos. Upon binding activated (processed) Spätzle, Toll dimerizes. The resulting conformational change allows the Toll TIR domain to bind to the TIR domain of the adaptor protein MyD88. (For simplicity, interactions and downstream events are shown for only one monomer of a dimeric Toll receptor). MyD88 brings along with it the adaptor protein Tube, an association mediated by a death domain in each protein. The bivalent Tube death domain then binds the death domain of Pelle, recruiting the inactive protein kinase into the signaling complex. Within the complex, Pelle undergoes autoactivation via autophosphorylation. Pelle then interacts with Dorsal-bound Cactus, an interaction enhanced by the binding of Dorsal to both Tube and Pelle. Pelle phosphorylates Cactus, triggering recognition by the Slimb β TrCP and subsequent ubiquitination and proteasome mediated degradation. Pelle likely also phosphorylates Dorsal, enhancing its gene regulatory activity upon release from Cactus, and Tube, initiating negative feedback regulation on Toll signaling.

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of the Toll ligand Spätzle can trigger a detectable level of Cactus degradation in the absence of Dorsal [42]. Consistent with these results, we find that Pelle preferentially phosphorylates signal responsive sites in Cactus *in vitro* in the absence of any other Toll pathway proteins.

Given that mammals require eight proteins in the portion of the Toll pathway that in flies is occupied by Pelle, it is appealing to consider the *Drosophila* pathway a model of simplicity. We note, however, that the history of Toll studies in flies and mammals is full of unexpected parallels as well as minor differences of major significance. Having offered the model shown in Figure 4, we will leave judgments regarding simplicity or complexity to others.

Materials and Methods

Molecular Biology

The GST Pelle- λ protein phosphatase (PPase) operon was generated by subcloning the λ -PPase gene from a pT7 vector (gift of Jack Dixon) into the Not I site of a pGEX 4T-1 vector (Amersham), and then inserting the Pelle coding sequence as an in-frame fusion with GST using EcoRI and XhoI restriction sites. The result was a 27 bp separation between coding regions of GST-Pelle and λ -PPase, with a Shine-Delgarno sequence 8 bases upstream of the phosphatase translational start site. For Cactus, PCR amplification was used to introduce an amino-terminal His₆ tag, with the tagged construct being inserted into the BamHI and

EcoRI restriction sites in the pRSET-A vector (Invitrogen). To generate the $\text{IkB}\alpha$ chimera, $\text{IkB}\alpha$ (a gift from Michael Karin) was fused with cactus Δ PEST and ligated into the pRSET-B vector (Invitrogen) in frame with the polyhistidine tag. All point mutations were generated by PCR SOEing [54].

S2 Cell Studies

S2 cells stably transfected with EGFR-Toll were treated with dsRNA and with mouse EGF, as described [24]. After 20 min, cells were harvested and 15 μg samples resolved on 8% SDS-PAGE and immunoblotted onto PVDF. Cactus was detected with a rabbit polyclonal antiserum (1:10,000), described previously [15].

Expression and Purification of Pelle

BL21 cells were transformed with Pelle- λ PPase construct and protein expression was induced as described [39]. BL21 cells transformed with the Pelle- λ PPase construct were grown for 5 hours at 28°C following IPTG induction. Cells from a 500 ml culture were pelleted and resuspended in 40 ml buffer containing 50 mM Tris HCl, pH 7.5; 2 mM EGTA; 0.5 M NaCl; and a protease inhibitor cocktail tablet (Roche). Cells were frozen and thawed; incubated with lysozyme, RNase, and DNase I in the presence of MgCl₂; and then lysed by three cycles of freezing (liquid nitrogen) and thawing (37°C). The lysate was spun at 25,000 g for 20 min at 4°C and the supernatant was decanted and incubated with 2 ml 50% glutathione Sepharose 4B resin slurry (GE Healthcare) in PBS for 2 hours at 4°C with gentle rocking. The beads were recovered by centrifugation at 500 g for 3 min at 4°C and the resin was washed three times in 10 ml PBS. GST-Pelle was eluted by adding 1 ml of elution buffer (10 mM glutathione, 50 mM Tris HCl, pH 8) and incubated for 1 hr at 4°C with gentle rocking.

Expression and Purification of His₆-cactus and Chimera

Wild-type and mutant forms of Cactus and the IkB -Cact Δ 144 chimera were each expressed with an amino-terminal His₆ tag in the pRSET vector in BL21 cells. Cactus proteins were renatured according to the protocol of Nüsslein-Volhard and colleagues [35]. Wild-type and mutant forms of Cactus and the IkB -Cact Δ 144 chimera were each expressed with an amino-terminal His₆ tag in the pRSET vector in BL21 cells. Expression was induced with 1 mM IPTG at 37°C for 3 hours. The lysate from a 500 ml culture was prepared by freeze/thaw cycles as described above and spun at 9000 g for 20 min. The pellet was resuspended in

20 ml buffer (20 mM Tris HCl, pH 8, 100 mM NaCl, 2 mM DTT, 2 mM EDTA, 1% Triton-X 100, 1/2 \times complete protease inhibitor tablet (Roche), stirred at 4°C for 1 hour, spun at 9000 g for 20 min, and resuspended in the same buffer without Triton-X 100. After incubation with stirring at 4°C for 1 hr, the preparation was pelleted and then resuspended to 10 mg/ml protein in urea buffer (20 mM Tris HCl, pH 8, 10 mM DTT, 1 mM PMSF, 8M urea) prior to renaturation.

Radiolabeling Assay of Pelle

GST-pelle (10 μg) was pre-incubated for 30 min at 30°C in 1 \times kinase buffer (10 μM ATP, 10 mM MgCl₂, 50 mM β -glycerophosphate, 25mM HEPES, pH 6.5) to allow activation by autophosphorylation. The activated Pelle (0.5 μg) was then incubated with recombinant His₆-Cactus (1.6 μg) in a volume of 40 μl in the presence of [γ -³²P]-ATP (13.3 μCi , Perkin Elmer) in 1 \times kinase buffer. Following reaction for 5 min at room temperature, samples were mixed with 10 μl 5 \times SDS loading sample buffer, boiled, and loaded (30 μl) on an 8% SDS protein gel. The gel was fixed twice for 30 min in fixing solution (50% methanol, 10% acetic acid) on a shaker, before being dried in a gel dryer for 1 hr. The gel was then exposed to a film and developed. Quantitation was performed with ImageJ software.

Immunoblot Assay of Pelle

Reactions were carried out as described above, except the kinase reactions were carried out with 11.2 μg of the chimera for 5 min at 30°C without radiolabel. Immunoblotting of the proteins transferred to a nitrocellulose membrane used rabbit anti- $\text{IkB}\alpha$ [pSpS^{32/36}] phosphospecific antibody (Invitrogen, 1:10,000) and α -rabbit secondary antibody (Sigma, 1:5,000), followed by detection using the Western Lightning-ECL chemiluminescence substrate (Perkin Elmer).

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Author Contributions

Conceived and designed the experiments: SW LK JD. Performed the experiments: LK JD. Analyzed the data: SW LK JD. Wrote the paper: SW LK.

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