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Reconstitution of Bacterial Tyrosine Kinase-Modulator Interaction in a Human Cell Line

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Many bacterial species express tyrosine kinases termed BY-kinases that share no homology with eukaryotic enzymes. We have previously reported that the *Staphylococcus aureus* BY-kinase CapB2 when fused with the C-terminal activation domain of its modulator CapA1, can translate into an active tyrosine kinase in HEK293T cells. In the present study, full-length CapA1 and CapB2 tagged with different fluorescent proteins were transfected into HEK293T cells. When expressed individually, the modulator CapA1, a membrane protein in bacteria, also appeared to localize to the cell membrane in HEK293T cells. In contrast, the catalytic subunit, CapB2, was found to be cytosolic. Coexpression of the two proteins resulted in apparent translocation of CapB2 to the membrane with concomitant activation of tyrosine kinase activity. This translocation and activation of CapB2 did not occur when the cytoplasmic C-terminal tail of CapA1 was deleted. Conversely, the CapA1 cytoplasmic C-terminal tail alone, when attached to a membrane localization sequence, was sufficient for CapB2 translocation and kinase activation. Our results indicate that the kinase activity of CapB2 is stimulated by direct interaction with the C-terminal cytoplasmic domain of CapA1 and that the process can be reconstituted and visualized in a human cell line. We created various mutants of CapA and CapB, and present data that demonstrate the correlation between CapA-CapB interaction and kinase activation.

Key words bacterial tyrosine kinase, protein-protein interaction, kinase activation

INTRODUCTION

Regulation of protein function by reversible phosphorylation is a mechanism widely spread in nature. In eukaryotes, a family of enzymes called Hanks-type protein kinases catalyze the phosphorylation on serine, threonine, and tyrosine residues. This family of enzymes also mediates serine/threonine phosphorylation in bacteria; however, conventional Hanks-type tyrosine kinases are not found in the bacterial genome, and tyrosine phosphorylation was once believed to be a regulation mechanism exclusive to eukaryotes. However, subsequent studies have unveiled the existence of distinctive tyrosine kinases that do not share homology with their eukaryotic counterparts. The largest and the best-studied family of such atypical tyrosine kinases is termed BY-kinases, characterized by nucleotide-binding Walker motifs and a C-terminal tyrosine cluster which is the site of autophosphorylation.[1–4)]

While BY-kinases are recognized for their essential role in the production and transport of capsular and extracellular polysaccharides,[5–7)] but they also participate in a wide range of events, including gene expression, stress responses, DNA metabolism, cell cycle, antibiotic resistance, spore formation, and biofilm formation.[3,8)] Deletion strains have been shown to display reduced virulence,[9–11)] and their lack of resemblance to eukaryotic counterparts makes BY-kinases attractive targets for antibacterial therapy.

The BY-kinase family comprises two subfamilies that differ in structure. Most Gram-positive bacterial BY-kinases comprise of two proteins encoded by two adjacent genes; a modulator subunit, which spans the membrane twice, and a cytosolic catalytic subunit. The catalytic subunit is activated upon interaction with the short cytoplasmic C-terminal tail of the modulator. In the majority of Gram-negative bacteria, the modulator and the catalytic domains exist within a single protein.

We have previously reported that the *Staphylococcus aureus* BY-kinase CapB2 when fused to the C-terminal domain of the modulator CapA, can be expressed as active tyrosine kinases in a human cell line HEK293T.[12)] The study identified several domains and residues essential for kinase activity. To gain more insight into the activation mechanism of Gram-positive bacterial BY-kinases, we investigated whether coexpression of the full-length BY-kinase catalytic and modulator subunits in mammalian cells can reproduce some phases of the protein interaction/kinase activation processes that occur in bacteria.

In the present study, we show that the expression of fluorescent protein-tagged modulator and catalytic subunits of BY-kinase in HEK293T cells enables visualization of the protein-protein interaction. Cells expressing BY-kinase proteins can be directly processed for immunoblotting analysis to assess kinase activation. We anticipate that the method will provide a convenient and efficient means to investigate BY-kinase acti-

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vation mechanisms and an approach for screening inhibitors.

MATERIALS AND METHODS

Antibodies and Plasmids Anti-phosphotyrosine antibodies 4G10 and PY20 were from Merck Millipore (U.S.A.) and Santa Cruz (U.S.A.), respectively. Monoclonal and polyclonal antibodies against green fluorescent protein (GFP) were from Medical & Biological Laboratories (Nagoya, Japan). The Gateway destination vector for emerald green fluorescent protein (EGFP)-tagged protein was from Invitrogen (U.S.A.). Destination vectors for mCherry- and DsRed-Express (DsRedEx)-tagged proteins with or without the N-terminal 10 amino acids of mouse Lck (LckN) for membrane localization[13] were created in our laboratory as previously described.[12] CapA1, CapA2, CapB1, and CapB2 DNAs were isolated by PCR from genomic DNA of *S. aureus* ATCC6538P and cloned into a pDONR221 vector to create Gateway entry clones. Mutations and deletions were introduced in entry clones using standard recombinant DNA technology. Genes were moved to destination vectors to generate expression plasmids according to the manufacturer's instructions.

Transfection and Immunoblotting To monitor expression and localization of proteins in mammalian cells, HEK293T cells were seeded in collagen-coated 96-well plates and transfected with 100 ng of expression plasmids as previously described.[12] Expression of bacterial protein was monitored and evaluated 24 h after transfection by imaging with a Keyence BZ-9000 fluorescence microscope with filter settings for GFP (for EGFP) or red fluorescent protein (for mCherry and DsRedEx). The cells were cultured for an additional 24 h and processed for immunoblotting as previously described.[12]

RESULTS

Interaction of the *S. aureus* CapA and CapB in HEK293T Cells The *S. aureus* genome codes for two tyrosine kinase catalytic subunits CapB1 and CapB2, as well as their cognate modulators CapA1 and CapA2. In a previous study,[12] we fused the C-terminal 29 amino acids of CapA1 (CapA1/CT) to CapB2 to create CapA1/CT-CapB2. By attaching fluorescent protein tags to the N-terminus, we demonstrated that this chimeric protein exhibits tyrosine kinase activity in HEK293T cells. In the present study, we fused full-length CapA1 to mCherry (Fig. 1A), and CapB2 was fused to EGFP (Fig. 1D), and the two proteins were transfected into HEK293T cells. While CapA1 has two stretches of hydrophobic amino acids that span the membrane in *S. aureus*, CapB2 does not contain any specific signal sequences and is a cytosolic protein. We expected that the two proteins would display similar localization when expressed in mammalian cells. Fluorescent microscopy revealed mCherry-CapA1 to be localized to the membrane (Fig. 2, left), whereas EGFP-CapB2 was distributed throughout the cytosol and the nucleus (Fig. 2, right).

We anticipated that the coexpression of CapA1 and CapB2 in HEK293T cells would result in CapB2 interacting with the cytoplasmic C-terminal tail of CapA1, with subsequent recruitment of CapB2 to the membrane. As Fig. 3A illustrates, when cotransfected with mCherry-CapA1, EGFP-CapB2 translocated from the cytosol to the membrane, and colocalization with mCherry-CapA1 was observed. These results indicate an interaction between the modulator and the catalytic subunits.

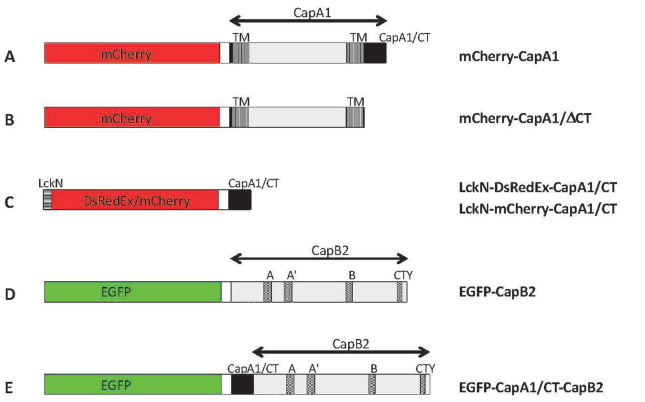

Fig. 1. Diagrams of the Representative Constructs Used in This Study A. mCherry-CapA1; B. mCherry-CapA1/ΔCT; C. LckN-DsRedEx-CapA1/CT and LckN-mCherry-CapA1/CT; D. EGFP-CapB2; E. EGFP-CapA1/CT-CapB2

LckN, N-terminal 10 amino acids of mouse Lck; TM, transmembrane domain; CapA/CT, CapA C-terminal cytoplasmic domain; Positions of Walker A (A), Walker A' (A'), Walker B (B), and C-terminal tyrosine cluster (CTY) are shown.

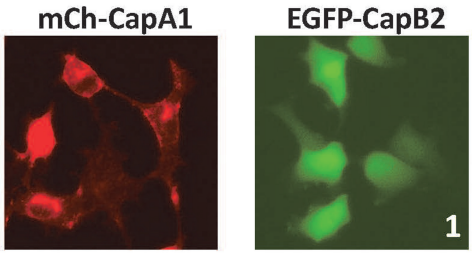

Fig. 2. Expression of *Staphylococcus aureus* CapA1 and CapB2 in HEK293T Cells

HEK293T cells were transfected with plasmids to express mCherry-CapA1 (mCh-CapA1) or EGFP-CapB2 (EGFP-CapB2). Images were captured after 24 h. The number on the lower right corner corresponds to the lane in Fig. 6.

We next examined whether removal of the cytosolic C-terminal tail of CapA1, which is the site of interaction, would abolish the translocation of the cotransfected CapB2 to the membrane. CapA1 C-terminal-tail-deletion mutant (CapA1/ΔCT) still contains the two membrane-spanning stretches (Fig. 1B) and was a membrane protein (Fig. 3B, left). Cotransfection of EGFP-CapB2 with CapA1/ΔCT resulted in the proteins remaining in the cytosol; translocation was not observed (Fig. 3B, right).

To further confirm that CapA1 and CapB2 interact, the last 29 amino acids of CapA1 was tagged with LckN-mCherry or LckN-DsRedEx (Fig. 1C), which the ten amino acid N-terminal dual acylation motif of mouse Lck directs the fused protein to the membrane.[13] As shown in Figs. 4A and 4B, EGFP-CapB2 colocalized with LckN-mCherry-CapA1/CT and LckN-DsRedEx-CapA1/CT at the membrane.

When CapB2 was fused with CapA1/CT and expressed as EGFP-CapA1/CT-CapB2 (Fig. 1E), it did not appear to interact with CapA1/CT (Fig. 4C, right). Presumably, the CapA binding regions of CapB2 are blocked by intramolecular interaction with the fused CapA1/CT and are no longer accessible to membrane-bound CapA1/CT.

Schematic drawings of EGFP-CapB2 coexpressed with mCherry-CapA1, mCherry-CapA1/ΔCT, and LckN-DsRedEx-CapA1/CT are shown in Fig. 5.

Interaction with CapA/CT Activates CapB2 Tyrosine Kinase We questioned whether interaction with CapA would

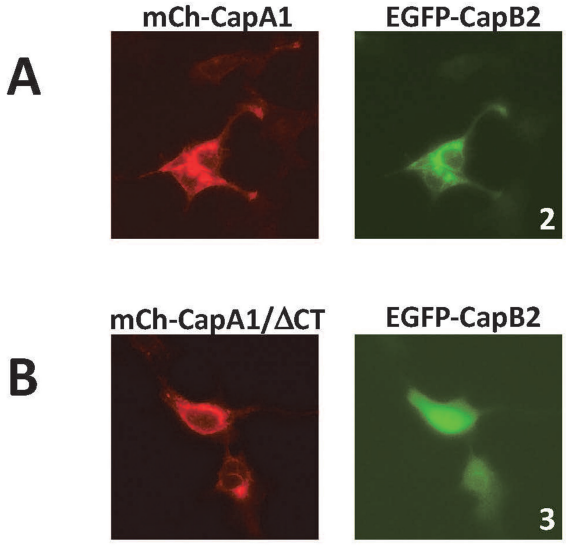

Fig. 3. Interaction of *Staphylococcus aureus* CapA1 and CapB2 in HEK293T Cells

HEK293T cells were transfected with indicated expression plasmids and photographed after 24 h. A. mCherry-CapA1 (mCh-CapA1) and EGFP-CapB2 (EGFP-CapB2); B. mCherry-CapA1/ΔCT (mCh-CapA1/ΔCT) and EGFP-CapB2. The numbers on the lower right corner correspond to the lanes in Fig. 6.

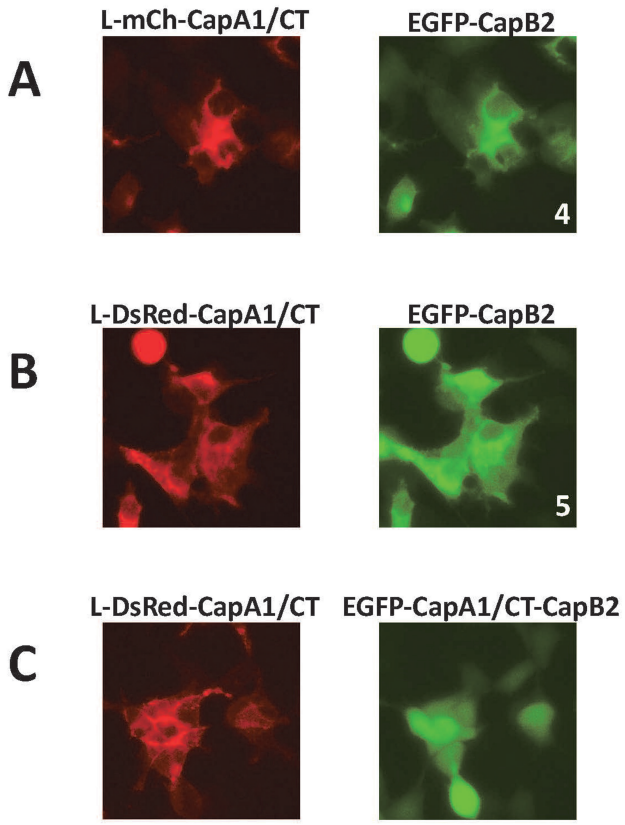

Fig. 4. CapB2 Interacts with C-terminal Cytoplasmic Domain of CapA1

HEK293T cells were transfected with indicated expression plasmids and photographed after 24 h. A. LckN-mCherry-CapA1/CT (L-mCh-CapA1/CT) and EGFP-CapB2; B. LckN-DsRedEx-CapA1/CT (L-DsRed-CapA1/CT) and EGFP-CapB2; C. LckN-DsRedEx-CapA1/CT and EGFP-CapA1/CT-CapB2. The numbers on the lower right corner correspond to the lanes in Fig. 6.

enhance CapB kinase activity in HEK293T cells. The cells transfected with EGFP-CapB2 in Figs. 2, 3, and 4 were processed and subjected to tyrosine phosphorylation analysis (Fig. 6).

EGFP-CapB2, by itself, showed modest autophosphorylation activity (lane 1). Its phosphotyrosine level clearly increased when transfected together with mCherry-CapA1 (lane 2), but not with mCherry-CapA1/ΔCT (lane 3). Lck N-mCherry-CapA1/CT (lane 4) and LckN-DsRedEx-CapA1/CT (lane 5) also enhanced tyrosine phosphorylation. Unexpectedly, cotransfection with LckN-DsRedEx-CapA1/CT resulted in the highest phosphotyrosine signal. Furthermore, in addition to EGFP-CapB2 (position shown by the arrow), LckN-DsRedEx-CapA1/CT also appeared to be tyrosine phosphorylated (position shown by the asterisk). These results indicate that the interaction of CapA1 and CapB2, and the subsequent activation of CapB2 kinase, can be reconstituted in HEK293T cells.

In our preceding report using CapA1/CT-CapB2 chimeras fused to LckN-DsRedEx,[12] we demonstrated that mutation of the ATP-binding lysine in the Walker A domain of CapB2 (K55M) completely abolished phosphorylation. On the other hand, replacement of the four C-terminal tyrosines reported to be the site of autophosphorylation (tyrosines 221, 222, 224, 225) with phenylalanines (4F) did not significantly alter the level of phosphotyrosine. Deletion of 20 amino acids from the N-terminus (ΔNT20) or 15 amino acids from the C-terminus (ΔCT15) had little effect, but the deletion of 34 amino acids from the N-terminus (ΔNT34) or 19 amino acids from the C-terminus (ΔCT19) significantly decreased phosphorylation. We made the corresponding mutants of CapB2 (Fig. 7A) tagged with EGFP. The EGFP-CapB2 mutants were cotransfected with LckN-DsRedEx-CapA1/CT, which had the most significant effect on activation of wild-type CapB2, and examined for their tyrosine phosphorylation and intracellular distribution.

The results of the phosphotyrosine blots were consistent with our previous observations using CapA/CT-CapB2 fusion proteins (Fig. 7B). Tyrosine phosphorylation of the K55M mutant was completely abrogated. The 4F, ΔCT15, and ΔNT20 mutants were still phosphorylated, but the phosphorylation of the ΔCT19 and ΔNT34 mutants decreased to the control level.

Fig. 7C shows the localization, i.e., the interaction with CapA1/CT of CapB2 mutants. The inactive K55M mutant translocated to the membrane, indicating that kinase activity is not a prerequisite for interaction with the modulator subunit. Other results were in good correlation with those of the phosphotyrosine blots. The 4F, ΔNT20, and ΔCT15 mutants were recruited to the membrane, but the ΔNT34 and ΔCT19 mutants with diminished tyrosine phosphorylation mainly remained cytosolic. Analogous results were obtained with full-length CapA1 tagged with mCherry instead of LckN-DsRedEx-CapA1/CT (data not shown).

We next made mutants of CapA/CT N-terminally tagged with LckN-DsRedEx, and coexpressed them with EGFP-CapB2 in HEK293T cells. Although it had been reported that CapA1 is a better activator of CapB2 than CapA2,[14,15] in the previous paper, we did not observe a clear difference between CapA1 and CapA2 regarding CapB2 activation effect.

The penultimate phenylalanine of CapA1 (204F) has been reported to directly interact with the adenine moiety of ATP.[16] The CapA1 sequence we used is that of *S. aureus* ssp. *aureus* 6850, in which the second-last amino acid is tyrosine (204Y) and not phenylalanine. Although we did not show the actual data, in the experiments with CapA1/CT-CapB2 chimeric pro-

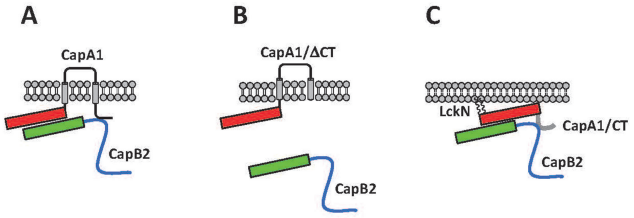

Fig. 5. Schematic Illustrations of Fluorescent Protein-Tagged CapA and CapB Proteins Expressed in Mammalian Cells

A. mCherry-CapA1 and EGFP-CapB2; B. mCherry-CapA1/ΔCT and EGFP-CapB2; C. LckN-DsRedEx-CapA1/CT29 and EGFP-CapB2

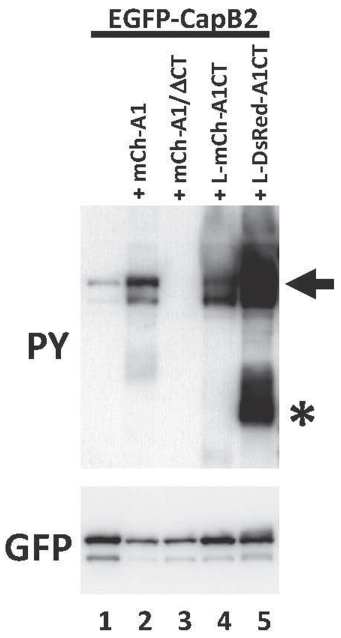

Fig. 6. C-terminal Cytoplasmic Domain of CapA1 Activates CapB2

HEK293T cells transfected with EGFP-CapB2 and CapA1 plasmids in Figs. 2 (lane 1), 3A (lane 2), 3B (lane 3), 4A (lane 4), and 4B (lane 5) were subjected to immunoblotting using anti-phosphotyrosine (PY) and anti-GFP (GFP) antibodies as described in Materials and Methods. Positions of EGFP-CapB2 (arrow) and LckN-DsRedEx-CapA1/CT (asterisk) are shown.

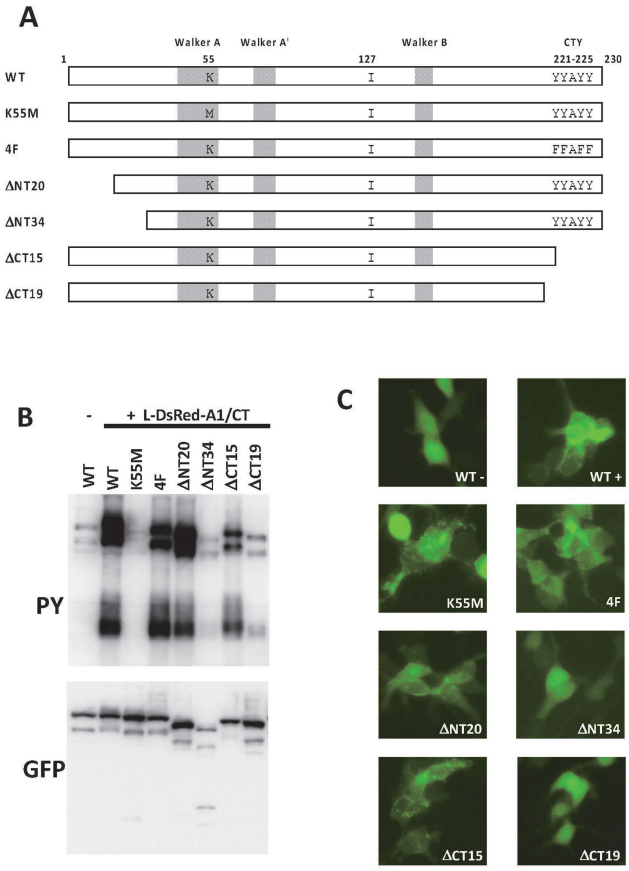

Fig. 7. Tyrosine Phosphorylation and Localization of Wild-type (WT) and Mutant EGFP-CapB2 Coexpressed with LckN-DsRedEx-CapA1/CT (L-DsRedEx-A1/CT) in HEK293T Cells

A. Diagrams of Wild-type (WT) and Mutant CapB2 used. Positions of lysine 55 and the C-terminal tyrosine cluster (CTY) are shown. The position of isoleucine 127, which was deleted in Fig. 9 is also shown. B. HEK293T cells transfected with LckN-DsRedEx-CapA1/CT and indicated EGFP-CapB2 plasmids were subjected to immunoblotting using anti-phosphotyrosine (PY) and anti-GFP (GFP) antibodies. C. Localization of EGFP-CapB2 mutants. HEK293T cells transfected with LckN-DsRedEx-CapA1/CT and indicated EGFP-CapB2 plasmids were photographed using filter settings for GFP.

teins, we did not observe a significant difference between the phenylalanine and the tyrosine versions.

In the present study, we compared the CapB2-activating effects of CapA1/CT(204Y), CapA1/CT(204F), and CapA2/CT (Fig. 8A). The three CapAs caused comparable activation of CapB2 (Fig. 8B, lanes 2, 3, and 6), reproducing our previous results using CapA/CT-CapB chimeras. Deletion of the last two amino acids of CapA1 (A1/CT-2aa) only slightly decreased tyrosine phosphorylation (Fig 8B, lane 4), indicating that phenylalanine or tyrosine at the penultimate position is not essential for interaction with CapB. Deletion of the last six amino acids (A1/CT-6aa) notably reduced tyrosine phosphorylation (Fig. 8B, lane 5) as well as interaction with CapB2 (Fig. 8C).

Activation of CapB1 by CapA/CT The *S. aureus* CapB1, despite its 70% similarity with CapB2, has been reported to be inactive as a kinase.[14–16] We cloned CapB1 with a deletion of valine 127 (CapB1/Δ127V), which, when fused to CapA1/CT, did not exhibit activity.[12] However, the insertion of valine (CapB1/127V) or isoleucine (CapB1/127I) at this position converted the catalytically inert protein into an active kinase. The deletion of the corresponding isoleucine 127 in CapB2 (CapB2/Δ127I) decreased, but did not completely abolish kinase activity. We tested whether comparable results would be obtained when CapA1/CT and CapB1 are expressed as separate proteins.

CapB1 and various mutants were fused to EGFP and cotransfected into HEK293T cells with LckN-DsRedEx-CapA1/CT. As shown in Fig. 9A, EGFP-CapB1/Δ127V largely remained cytosolic, indicating that its interaction with CapA1/CT is significantly impaired. In contrast, CapB1/127V, CapB1/127I and CapB2/Δ127 exhibited more obvious membrane translocation. Their mutants of the ATP-binding lysine (K55M) were also recruited to the membrane, supporting that kinase activity is not required for interaction with the modulator subunit.

Analysis of tyrosine phosphorylation in transfected cells revealed that CapB1/Δ127I was not tyrosine phosphorylated (Fig. 9B, lane 1). CapB1/127V (lane 2), CapB1/127I (lane 4), and CapB2/Δ127I (lane 7), although to a lesser extent than CapB2 (lane 6), was activated by CapA1/CT, and mutation of the ATP-binding lysine to methionine completely abolished phosphorylation (lanes 3 and 5). These results were expected

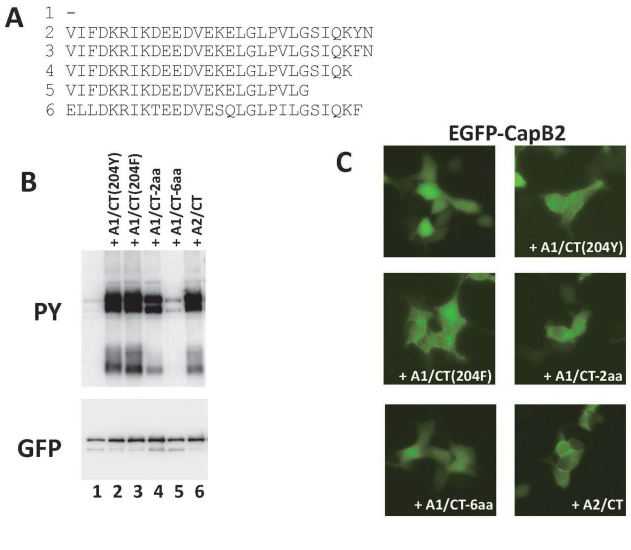

Fig. 8. Effects of LckN-DsRedEx-tagged-CapA C-terminal Domains on Activation and Localization of CapB2 in HEK293T Cells

A. Amino acid sequences of CapA C-terminal domains used. Numbers 1 to 6 correspond to the lanes in B. B. CapB2 tyrosine phosphorylation. HEK293T cells were transfected with plasmids to express EGFP-CapB2 and indicated CapA C-terminal domains tagged with LckN-DsRedEx and subjected to immunoblotting using anti-phosphotyrosine (PY) and anti-GFP (GFP) antibodies. C. Localization of EGFP-CapB2. HEK293T cells transfected with EGFP-CapB2 and indicated LckN-DsRedEx-CapA/CT plasmids were photographed using filter settings for GFP.

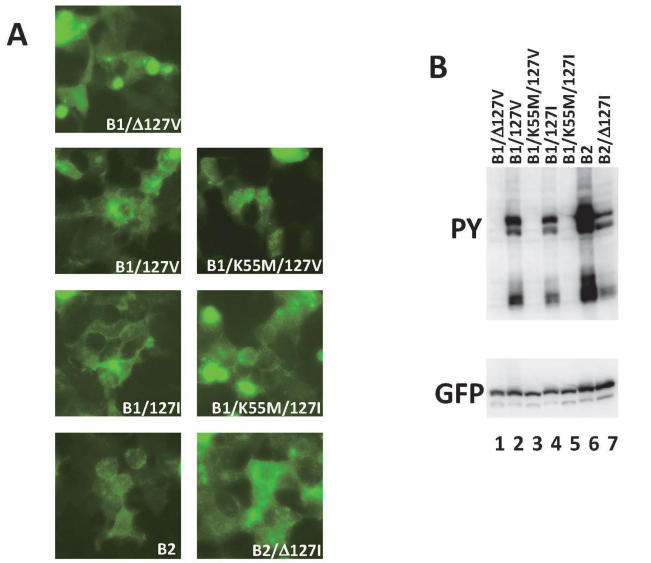

Fig. 9. Localization and Tyrosine Phosphorylation of Wild-type and Mutant EGFP-CapB1 and EGFP-CapB2 Coexpressed with LckN-DsRedEx-CapA1/CT in HEK293T Cells

A. HEK293T cells transfected with LckN-DsRedEx-CapA1/CT and indicated EGFP-CapB plasmids were photographed using filter settings for GFP. B. HEK293T cells transfected with LckN-DsRedEx-CapA1/CT and indicated EGFP-CapB plasmids were subjected to immunoblotting using anti-phosphotyrosine (PY) and anti-GFP (GFP) antibodies.

and are in excellent agreement with our previous observations of CapA/CT-CapB fusion proteins. Furthermore, essentially identical data were obtained using LckN-DsRedEx-CapA2/CT (data not shown). The results suggest that the reason for the inactivity of CapB1/Δ127V is due, in part, to decreased affinity with the CapA C-terminal activation domain.

DISCUSSION

Gram-positive bacterial BY-kinases are activated by interaction of the cytosolic catalytic subunit with the membrane modulator subunit. In the present study, we showed that the interaction-activation process can, at least in part, be reconstituted in a mammalian cell line. The interaction is visualized through recruitment of the fluorescent protein-tagged kinases to the membrane, and activation can be assessed by subsequent immunoblotting.

Tyrosine phosphorylation of EGFP-CapB2 was particularly prominent when expressed with LckN-DsRedEx-CapA1/CT. Compared to full-length CapA1 (205 amino acids), the 29-amino acid tail was expressed at a much higher level, judging from immunoblotting using anti-RFP antibody (data not shown). It is not clear why LckN-DsRed-CapA1/CT was far more effective in terms of CapB2 activation than LckN-mCherry-CapA1/CT (Fig. 6, lanes 4 and 5). Whereas mCherry is a monomeric protein, DsRedEx forms a tetramer and may enable a more efficient protein-protein interaction.

BY-kinases have been postulated to possess loose substrate specificity,[4] and phosphorylated proteins that are not usually substrates of tyrosine kinases, such as glutathione S-transferase and maltose-binding protein.[6] We have shown that CapA1/CT-CapB2 fused to LckN-DsRedEx not only autophosphorylated on the C-terminal tyrosine cluster but also phosphorylated the fluorescent protein tag attached.[12] In the present experiments, mutants of the CapB2 C-terminal tyrosine cluster (4F and ΔCT15) were still tyrosine phosphorylated implying that they phosphorylate the tagged EGFP. Moreover, the coexpressed LckN-DsRedEx-CapA/CT proteins were also phosphorylated. The 29-amino acid tail of CapA1 we mainly used (CapA1/CT) contains one tyrosine residue (tyrosine 204), but CapA1/CT(204F), CapA1/CT-2aa and CapA2/CT do not (Fig. 8), and it is likely that in Figs. 6, 7, and 8, CapBs phosphorylated itself, EGFP fused on the N-terminus, and DsRedEx fused to CapA/CTs.

CapB1 shares over 70% homology with CapB2, but its kinase activity had not been detected.[14–16] The CapB1 we originally cloned had a deletion of valine 127 (CapB1/Δ127V). In agreement with previous reports, this version of CapB1 did not display detectable kinase activity when fused to CapA1/CT. Unexpectedly, however, insertion of valine or isoleucine at amino acid position 127 converted the catalytically inert protein into an active kinase. In line with previous studies, in the present experiments, CapB1/Δ127V did not show any tyrosine kinase activity. Interestingly, CapB1/Δ127V showed reduced interaction with CapA1/CT. Insertion of the missing amino acid not only restored kinase activity, but also the interaction with CapA/CT.

CapB1/Δ127V was the only sequence found from the strain we used, and CapB1 may not actually function as a kinase in bacteria. Nevertheless, our study highlights the importance of the region surrounding amino acid 127 in CapA-CapB interaction. The proximity of this residue is well conserved among many bacteria.[4] The contribution of this region, in conjunction with other domains, to the interaction with CapA is yet to be elucidated.

Various methods have been employed to analyze the interaction of the BY-kinase modulator and catalytic subunits, including bacterial and yeast two-hybrid systems,[17–21] immu-

noprecipitation of *in vivo*[17] or *in vitro* cross-linked proteins,[14,22] and expression of fluorescent-tagged proteins in bacteria.[20] The use of these methods greatly contributed to the understanding of BY-kinase activation mechanisms. However, the procedures can be time- and labor-intensive, especially when a large number of samples are to be examined.

Although the heterologous expression system described here does have limitations, it also offers unique advantages. There is no need to establish special bacterial strains or mammalian cell lines. Cells can be seeded into microtiter plates and transfected for expression of bacterial proteins. Visual analysis is much easier in large mammalian cells than in bacteria, and after observation, cells are directly processed for immunoblotting and examined for tyrosine phosphorylation. Protein purification is not necessary. These features enable quick testing of multiple mutants in various combinations, and we anticipate that the use of our method will contribute to the clarification of the mechanism of BY-kinase activation and discovery of inhibitors.

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Conflict of interest The authors declare no conflict of interest.

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