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The *Pseudomonas aeruginosa* lectin LecA triggers host cell signalling by glycosphingolipid-dependent phosphorylation of the adaptor protein CrkII

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Abstract

The human pathogen *Pseudomonas aeruginosa* induces phosphorylation of the adaptor protein CrkII by activating the non-receptor tyrosine kinase Abl to promote its uptake into host cells. So far, the bacterial as well as host cell factors which induce Abl/CrkII signalling are entirely unknown. In this research, we employed human lung epithelial cells H1299, Chinese hamster ovary cells and *P. aeruginosa* wild type strain PAO1 to study the invasion process of *P. aeruginosa* into host cells by using microbiological, biochemical and cell biological approaches such as Western Blot, immunofluorescence microscopy and flow cytometry. Here, we demonstrate that the host glycosphingolipid globotriaosylceramide, also termed Gb3, represents a signalling receptor for the *P. aeruginosa* lectin LecA to induce CrkII phosphorylation at tyrosine 221. Alterations in Gb3 expression and LecA function correlate with CrkII phosphorylation. Interestingly, phosphorylation of CrkII^{Y221} occurs independently of Abl kinase. We further show that Src family kinases transduce the signal induced by LecA binding to Gb3, leading to Crk^{Y221} phosphorylation. In summary, we identified LecA as a bacterial factor, which utilizes a so far unrecognized mechanism for phospho-CrkII^{Y221} induction by binding to the host glycosphingolipid receptor Gb3. The LecA/Gb3 interaction highlights the potential of glycolipids to mediate signalling processes across the plasma membrane and should be further elucidated to gain deeper insights into this non-canonical mechanism of activating host cell processes.

Keywords

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Running title

LecA binds to Gb3 and phosphorylates CrkII

1. Introduction

Pseudomonas aeruginosa is a Gram-negative opportunistic human pathogen, which causes severe infections of the respiratory tract, urinary tract, skin and eyes [1]. Moreover, *P. aeruginosa* causes chronic infections in the lungs of patients with cystic fibrosis (CF) leading to damage of the lung epithelium, decline in lung function and death [2]. Internalisation of *P. aeruginosa* by host cells significantly contributes to its pathogenicity [3-5]. *P. aeruginosa* interacts with various host cell receptors including $\alpha 5\beta 1$ integrin [6], CF transmembrane conductance regulator (CFTR) [7] and the glycosphingolipids (GSLs) asialo GM1 and asialo GM2 [8-10], to ensure efficient adhesion and cell entry. Although asialo GM1 and asialo GM2 have been shown not to be directly involved in initial Type-4-pili-mediated adhesion [11], it is widely accepted that host cell GSLs can be hijacked by microbial factors leading to signalling and internalisation events [12]. However, the interactions of *P. aeruginosa* ligands with GSLs and their pathophysiological consequences are poorly understood. In recent years, the two *P. aeruginosa* lectins LecA and LecB have attracted attention as drug targets, also because of exciting new discoveries about their diverse roles in impairing host cell physiology [13-20]. LecA (also known as PA-IL) is a homotetrameric, multifunctional virulence factor with a preferential binding affinity for α -D-galactosylated glycans, in particular for the GSL globotriaosylceramide (galactose $\alpha 1-4$ galactose $\beta 1-4$ glucosyl ceramide, also known as Gb3, CD77 or Pk blood group antigen) [21-26]. It is involved in the attachment of the bacterium to human lung epithelial cells and biofilm formation [22, 27-29]. Furthermore, LecA induces alveolar destruction during bacterial infection *in vivo* [28]. Recently, we have demonstrated that LecA acts as an invasion factor for *P. aeruginosa* besides its known role as an adhesion factor. Thereby, LecA interacts with Gb3, triggering membrane engulfment of *P. aeruginosa* at the initial stage of entry into human lung epithelial cells [17]. So far, it is unknown whether LecA further acts on other stages of the invasion process, *e.g.* the induction of host cell signalling.

Crk (CT10 regulator of kinase) family members, including CrkI, CrkII and Crk-like, serve as major signalling integrators in multiple cellular processes, such as cell adhesion, migration, and immune responses [42]. Amongst others, Crk adapter proteins are major substrates for the Abelson non-receptor tyrosine kinase Abl, but become also phosphorylated by membrane-bound receptors like EGFR and integrins [41, 76, 77]. The family of Abelson non-receptor tyrosine kinases consists of two widely expressed members, Abl (*ABL1*) and Arg (Abl-related gene; *ABL2*) [35, 40]. Abl acts in conjunction with Crk to regulate a number of cellular processes, including cell growth, survival, stress responses, proliferation, adhesion, actin dynamics and cell migration [35-39]. It is known that Abl/CrkII interaction is often engaged by human pathogens like *Shigella flexneri* and *Salmonella enterica*, for cell invasion, actin tail formation and host cell release [31-34]. Data of a previously conducted RNAi screen using *Drosophila* S2 cells demonstrate that Abl-dependent CrkII phosphorylation promotes the cellular uptake of *P. aeruginosa* [30]. However, the bacterial factors as well as the host cell receptors, which activate Abl/CrkII signalling during *P. aeruginosa* invasion, remain elusive.

In our study, we identified the host cell GSL Gb3 and the bacterial lectin LecA as key factors for CrkII^{Y221} phosphorylation during infection of human lung epithelial cells by *P. aeruginosa*.

Of note, we could demonstrate that the phosphorylation of CrkII is not depending on the presence and activity of Abl kinase. Binding of LecA to Gb3 is probably sensed by c-Src, which mediates the phosphorylation of CrkII, as suggested by Src kinase inhibitor studies.

2. Results

2.1 *P. aeruginosa* entry into lung epithelial cells is sensitive to tyrosine kinase inhibitors

In their study, Pielage *et al.* demonstrated that Abl-dependent CrkII phosphorylation supports the cellular internalisation of *P. aeruginosa* into HeLa and 3T3 fibroblast cells [30]. Therefore, we first explored whether the uptake of the *P. aeruginosa* strain PAO1 into our cell model, the human lung epithelial cell line H1299, is susceptible to tyrosine kinase inhibitors, which have been described to inhibit Abl kinase activity. We used imatinib (STI571, Gleevec®), a well-known Abl kinase inhibitor [43, 44], and quantified the uptake of PAO1 into H1299 cells in the presence of the inhibitor. Imatinib lowered PAO1 invasion in a dose-dependent manner (Fig. 1A). For instance, a reduction of around 80 % in invasiveness was achieved at a concentration of 100 µM. In a complementary approach, we used nilotinib (AMN107), which is a 20- to 40-fold more potent Abl kinase inhibitor [45-49]. Nilotinib treatment resulted in an even more pronounced inhibition of PAO1 invasion into H1299 cells (Suppl. Fig. 1A). A concentration of 1 µM of nilotinib was already sufficient to decrease PAO1 host cell entry by 50 %, whereas the reduction in invasion at a concentration of 100 µM was comparable to that achieved with imatinib.

To assess whether Abl kinase is also activated during infection of lung epithelial cells by PAO1, we analysed lysates of PAO1-infected H1299 cells for the phosphorylation level of CrkII at tyrosine 221 (phospho-CrkII^{Y221}), which has been described to be phosphorylated by Abl kinase [41]. We observed that the phospho-CrkII^{Y221} level was significantly increased in H1299 cells infected with PAO1 compared to uninfected cells (Fig. 1B). We next investigated if one of the virulence factors of *P. aeruginosa*, the lectin LecA, contributes to CrkII^{Y221} phosphorylation. LecA is a multifunctional virulence factor, supporting biofilm formation, bacterial attachment and cellular invasion [50-54, 17]. Our previous data adumbrates a role of LecA in signalling-dependent entry processes. Therefore, we used a LecA-deletion mutant of PAO1 (Δ lecA, Suppl. Fig. 1B) to test if LecA is involved in CrkII phosphorylation during infection. Interestingly, Δ lecA-infected H1299 cells showed a lower phospho-CrkII^{Y221} level than wild type PAO1-infected cells, indicating that LecA might play a role in CrkII phosphorylation (Fig. 1B). However, probably due to other activating bacterial factors, which could partially compensate the LecA-deletion effect, the level of phospho-CrkII^{Y221} in Δ lecA-infected H1299 cells did not approach the basal level. These results prompted us to analyse the impact of LecA in more detail in the absence of the multifactorial, bacterial context.

2.2 *P. aeruginosa* lectin LecA triggers CrkII^{Y221} phosphorylation in human lung epithelial cells

We then aimed for specifically addressing the effect of LecA on CrkII phosphorylation in the absence of additional, putative bacterial activators. Therefore, we used purified LecA for stimulation experiments of H1299 cells. Indeed, incubation of cells with nanomolar concentrations (20-100 nM) of LecA led to a significant increase in CrkII^{Y221} phosphorylation (Fig. 2A). Time-resolved CrkII^{Y221} phosphorylation studies showed a remarkable induction of phosphorylation, which peaked at 60 min of LecA treatment (Fig. 2B). Subsequent experiments using imatinib and nilotinib, demonstrate the dependency of LecA-induced phosphorylation of CrkII^{Y221} on general tyrosine kinase activity (Fig. 2C and Suppl. Fig. 2A). Both inhibitors

potently reduced CrkII^{Y221} phosphorylation, for example by up to 90 % at a concentration of 1 μ M of nilotinib. Besides imatinib and nilotinib, we tested the effect of two other tyrosine kinase inhibitors on LecA-induced CrkII^{Y221} phosphorylation: dasatinib (BMS-354825) and GNF-2. Dasatinib is a highly potent ATP-competitive dual Src/Abl kinase inhibitor [55], which is thought to bind the active conformation of the Abl kinase domain [49, 56]. GNF-2, which binds to the myristate-binding pocket in the C-lobe of Abl kinase domain, is not known to inhibit any additional kinases [57-59]. The same level of inhibition was obtained with dasatinib and GNF-2 (Suppl. Fig. 2B).

To explore whether the binding of LecA to glycosylated receptors [21-23] is crucial for CrkII phosphorylation, we pre-incubated LecA with *p*-nitrophenyl- α -D-galactopyranoside (PNPG), a soluble galactose, which competes with galactose-conjugated host cell receptors for LecA binding. PNPG pre-treatment of LecA inhibited binding of LecA to H1299 cells in a dose-dependent manner as confirmed by fluorescence microscopy and fluorescence-activated cell sorting (FACS) (Suppl. Fig. 2C-D). Consequently, PNPG prevented CrkII phosphorylation by LecA in a concentration-dependent manner as indicated by a significant reduction of phospho-CrkII^{Y221} levels (Fig. 2D). Collectively, these results suggest that CrkII phosphorylation in PAO1-infected lung epithelial cells is triggered by binding of LecA to glycosylated host cell receptors.

2.3 Gb3 binding is crucial for LecA-induced CrkII phosphorylation

LecA has been reported to bind the α -D-galactose of the host GSL Gb3 [24, 25, 53]. Therefore, we explored if GSLs in general and Gb3 in particular could serve as lipid receptors for LecA-triggered activation of Abl kinase. To this end, we inhibited the synthesis of glucosylceramide, a precursor of GSLs, in H1299 cells, using DL-*threo*-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol (DL-PPMP) [9, 60]. Treatment of H1299 cells with DL-PPMP for seven days prior to LecA incubation completely prevented LecA-induced phosphorylation of CrkII^{Y221} (Fig. 3A).

To deplete Gb3 specifically from the plasma membrane, H1299 cells were incubated with different concentrations of the B-subunit of Shiga toxin (StxB) to induce Gb3 endocytosis [61-64]. Subsequent incubation of cells with Cy3-labeled StxB confirmed the dose-dependent depletion of Gb3 (Suppl. Fig. 3A, left panel), whereas the level of phospho-CrkII^{Y221} was not altered (Suppl. Fig. 3A, right panel). Interestingly, LecA treatment could not raise the amount of phospho-CrkII^{Y221} beyond the basal level, when cells were pre-treated with 65 nM StxB (Fig. 3B, indicated by a horizontal line).

To prove that Gb3 is indeed the key receptor for LecA-mediated CrkII phosphorylation, we used Chinese hamster ovary (CHO) cells, which do not express Gb3 [65]. Accordingly, LecA incubation did not change the phospho-CrkII^{Y221} levels in these cells (Suppl. Fig. 3B). However, when CHO cells were stably transfected with Gb3 synthase (CHO-Gb3) (Suppl. Fig. 3C), they were sensitized for LecA-dependent phosphorylation of CrkII^{Y221} in contrast to cells which were transfected with the empty vector (CHO-vector) (Fig. 3C). Taken together, our data suggest that LecA induces CrkII phosphorylation by binding to the host GSL Gb3. We subsequently analysed if Abl kinase is responsible for LecA-mediated Crk^{Y221} phosphorylation.

2.4 Abl kinase is not involved in LecA-triggered CrkII^{Y221} phosphorylation

Since CrkII is reported to be a substrate of Abl, we asked whether Abl is involved in LecA-triggered phosphorylation of CrkII. Therefore, we performed an Abl knock down by RNAi. siRNA-mediated knock down resulted in up to 90 % decreased levels of Abl compared to cells

transfected with scrambled siRNA. Interestingly, LecA was still able to stimulate CrkII phosphorylation in Abl knock down cells, with even increased levels compared to cells transfected with scrambled siRNA (Fig. 4). These results demonstrate that LecA triggers CrkII phosphorylation in an Abl-independent manner. In conclusion, the tyrosine kinase inhibitors that prevented LecA-triggered phosphorylation of CrkII (Fig. 2, Suppl. Fig. 2A, B) seem to affect also other tyrosine kinases beyond Abl, which are involved in CrkII^{Y221} phosphorylation, e.g. Src family kinases.

2.5 Src kinases mediate LecA-induced Abl-independent CrkII phosphorylation in human lung epithelial cells

Lectin/toxin binding to glycosphingolipids has been linked to the activation of Src family kinases (SFKs) and SFK-mediated signalling in mammalian cells [66-68]. SFKs, including Src, Fyn, Yes, Blk, Yrk, Fgr, Hck, Lck and Lyn, are involved in apoptosis, cell cycle progression, cytoskeletal rearrangement, differentiation, development, immune response, nervous system function, and transcription [69, 70]. We tested whether Src kinases play a role in LecA-triggered CrkII phosphorylation in human lung epithelial cells. Therefore, we used a highly selective SFK inhibitor (KB SRC4), which does not inhibit homologous kinases, such as Abl kinase, in contrast to other SFK inhibitors [71]. Treatment of H1299 cells with KB SRC4 prevented LecA-induced phosphorylation of CrkII^{Y221} completely at 10 μ M (Fig. 5A), indicating that Src kinases are crucial upstream factors for CrkII^{Y221} phosphorylation.

Based on these findings, we then specifically tested the phosphorylation of Src at Y418, which is required for full Src activity. LecA increased phospho-Src^{Y418} levels in H1299 cells (Fig. 5B) characterizing Src as a signalling mediator amongst other kinases for CrkII phosphorylation. Pre-incubation of cells with Nilotinib prevented CrkII phosphorylation, as observed in previous experiments (Suppl. Fig. 1A), but did not affect LecA-induced Src kinase phosphorylation (Fig. 5C). In summary, our data suggest that the host GSL receptor Gb3 is engaged by *P. aeruginosa* through its lectin LecA to activate c-Src, which acts upstream or in parallel with Nilotinib-sensitive kinases to phosphorylate CrkII^{Y221}, and to promote bacterial entry into human lung epithelial cells.

3. Discussion

Abl kinase function is often coupled to CrkII phosphorylation during infection of different human pathogens. However, the molecular mechanisms leading to activation of the Abl/CrkII pathway including pathogenic factors are largely unknown. For instance, Abl regulates the cellular invasion of the Gram-negative bacteria *S. flexneri* and *S. enterica* serovar Typhimurium via phosphorylation of CrkII [31, 34]. The type III secretion systems (T3SS) of these two bacteria are required for host cell invasion by manipulating the actin polymerization machinery, involving the Arp2/3 and N-WASP/WAVE complexes in an Abl-dependent manner [32, 34, 72]. However, it is largely unknown whether (effector) proteins, translocated through the T3SS, could directly activate Abl kinase or represent Abl substrates. Solely for the effector proteins CagA of *Helicobacter pylori* and Tir of enteropathogenic *Escherichia coli* (EPEC), it was shown that they represent Abl kinase substrates [73, 74]. A recent study identified Abl tyrosine kinase coupled to CrkII phosphorylation to be an essential host cellular pathway for *P. aeruginosa* internalisation [30]. It is not known which bacterial factor triggers the activation of Abl-dependent CrkII phosphorylation during the invasion process of *P. aeruginosa*. Our study highlights that LecA induces phospho-CrkII^{Y221} by binding to the GSL Gb3 in the host cell plasma membrane. Since

there is currently no evidence that LecA is directly translocated into the cytoplasm of host cells or represents a substrate for host cell kinases, LecA could either function at the host cell surface or upon endocytosis. This certainly represents an uncommon mode of activation amongst Gram-negative pathogens.

Proteinaceous membrane receptors, including EGFR, PDGFR, GPCR and β 2-integrins, have been described to be implicated in Abl activation and/or direct phosphorylation of CrkII [66, 75-77]. Here, we demonstrate that the GSL Gb3 mediates LecA-dependent CrkII activation (Fig. 3 and Suppl. Fig. 3), and therefore provides an uncommon and as yet undescribed receptor for a pathogenic bacterium to induce host cell signalling. The molecular mechanism how Gb3, which is located in the outer leaflet of the plasma membrane, communicates with the cytosolic membrane leaflet and induces CrkII phosphorylation on the cytosolic side is still unclear. Previous studies have shown that asymmetrically distributed GSLs with long fatty acyl chains might lead to interdigitation into the opposing leaflet of the bilayer [78, 79]. A recent study demonstrates that lactosyl ceramide (LacCer) species, which are common components of GSLs including Gb3, are indispensable for activation of the sarcoma tyrosine-protein kinase Lyn and that especially long fatty acid chains promote Lyn kinase activation [80, 81]. In fact, binding of StxB to Gb3 has been shown to activate Lyn kinase [82]. Other groups have shown that differently hydroxylated Gb3 species have varying affinity for StxB, which consequently lead to differences in Src kinase activation [83, 84]. Src kinases themselves have been reported to regulate Abl kinase or Abl-dependent Crk phosphorylation [66, 68] and notably, it has also been demonstrated for Lyn to promote cell entry of *P. aeruginosa* [85, 86]. In light of our observation of a LecA-induced and SFK-mediated CrkII phosphorylation, it will be interesting to investigate if distinct Gb3 species (in terms of hydroxylation, fatty acid chain length and saturation) determine the recruitment and activation of specific SFKs to promote bacterial uptake. Interestingly, although StxB and LecA both bound to Gb3, only LecA induces CrkII phosphorylation. Therefore, in addition to the diverse Gb3 species mentioned above, differences in the avidities between StxB and LecA towards Gb3 may lead to differences in Gb3 clustering behavior and receptor complex geometries in the plasma membrane, which could be decisive for recruiting and activating SFKs upstream of CrkII. Of note, we demonstrate here that CrkII is phosphorylated independently of Abl kinase but sensitive to Nilotinib treatment. This raises the question, which kinase(s) is/are acting upstream. Amongst those kinases that are effectively inhibited by nilotinib beyond c-Abl, are receptor tyrosine kinases [87]. These kinases, such as the Discoidin domain receptor (DDR) [88], have been reported to specifically bind CrkII, and could therefore be interesting candidates to be further explored if they phosphorylate CrkII in a Gb3-dependent manner.

Previous studies revealed the multiple functions of LecA as a virulence factor of *P. aeruginosa*, which is involved in bacterial invasion, adhesion and biofilm formation [17, 28, 53, 54]. Our observation of LecA-induced CrkII activation adds a previously unidentified role of this lectin as a bacterial signalling factor, which regulates the entry of *P. aeruginosa* into host cells (Suppl. Fig. 4). These diverse roles of LecA are even more striking because LecA has no enzymatic activity, which could be attributed to a certain function.

Considering the multifactorial, cellular context of *P. aeruginosa*, LecA might not be the only lectin which triggers Abl kinase activity in lung epithelial cells. In fact, ongoing experiments in our lab with the fucose-binding lectin LecB, another lectin expressed by *P. aeruginosa*, show an elevating effect of LecB on phospho-CrkII^{Y221} levels in lung epithelial cells (data not shown), but neither the host cell receptor(s) nor the molecular mechanism of Abl activation have been solved

yet. Moreover, the fimbrial adhesin PapG of uropathogenic *E. coli*, which, like LecA, also binds to a galactose-containing glycolipid (α-D-galactopyranosyl-(1→4)-3-D-galactopyranose) [89] might be an interesting candidate to conceptually prove the potential of GSL-binding lectins to trigger CrkII signalling in epithelial cells.

Our data suggest a new signalling receptor for *P. aeruginosa* invasion and demonstrates a bivalent role for LecA during the bacterial invasion process. LecA-Gb3 interactions not only trigger initial, bacterial membrane engulfment [17] but may also induce CrkII-dependent signalling that likely promotes efficient bacterial entry into host cells. Hence, this lectin-GSL complex may represent a potential target for drug development. Synthetic, bivalent LecA ligands have already been shown to have a pronounced inhibitory effect on bacterial uptake at low micro-molar concentrations [18], probably reflecting the significant impact of this lectin on *P. aeruginosa* uptake. On the other hand, the identification of host cell factors, like Gb3, c-Src and CrkII, offers promising approaches for the development of drugs, targeting distinct host cell processes in order to face the problem of increasing antibiotic-resistant pathogens. The second generation tyrosine kinase inhibitor nilotinib, which is a well-tolerated drug and 20- to 40-fold more potent compared to imatinib [45-49], might prove to be effective against *P. aeruginosa* and other pathogens that subvert GSL/tyrosine kinase-dependent pathways.

4. Materials and Methods

4.1 Cell Culture, generation of stable cell lines and depletion of GSLs

The human lung epithelial cell line H1299 (American Type Culture Collection No. CRL-5803) was grown in Roswell Park Memorial Institute (RPMI) 1640 medium. CHO cells were grown in Dulbecco's Modified Eagle Medium (DMEM) medium. For the generation of stable cell lines, CHO cells were transfected with a plasmid, either encoding Gb3-synthase (*Homo sapiens* alpha 1,4-galactosyltransferase, NCBI reference sequence: NM_017436.5, expressed from vector pcDNA3; “CHO-Gb3”) or the empty vector (“CHO-vector”, Clontech Cat. No. 632523), both containing a Geneticin resistance marker. After selection with 2 mg/ml Geneticin, positive clones were maintained in DMEM containing 1 mg/ml Geneticin. To check for the expression of Gb3, we used Alexa488-labeled B-subunit of Shiga toxin (StxB-Alexa488), which is known to interact specifically with the GSL Gb3. All media were supplemented with 10 % fetal calf serum (FCS) and 2 mM L-glutamine. For GSL depletion, H1299 cells were passaged for seven days in the presence of 5 μM of glucosylceramide synthase inhibitor DL-PPMP. All cells were grown at 37 °C and 5 % CO₂.

4.2 Bacterial strains and infections

The *P. aeruginosa* PAO1 wild type strain (PAO1), the LecA deletion mutant of PAO1 (Δ *lecA*) and GFP-tagged PAO1 [17] were grown on Luria Broth (LB) agar plates and in LB medium. GFP tagging and depletion of *lecA* in *P. aeruginosa* were described in detail in Eierhoff *et al.* [17]. The GFP-tagged strain was cultured in the presence of Gentamicin (60 μg·ml⁻¹). For infection, overnight cultures of *P. aeruginosa* were grown in LB medium to stationary phase. H1299 cells, CHO-vector cells and CHO-Gb3 cells were seeded in 6-well plates in growth medium. Next day, cells (70-80 % confluent) were starved in growth medium without FCS for 2 h at 37 °C. Then, cells were incubated with *P. aeruginosa* for 60 min at 37 °C at a multiplicity of infection (MOI) of 100. Cells were then lysed, and lysates were processed for Western blot analysis.

4.3 Invasion assays

Overnight cultures of GFP-tagged *P. aeruginosa* PAO1 were pelleted and re-suspended in RPMI medium, containing additionally 1 mM CaCl₂ and MgCl₂. H1299 cells (70-80 % confluent) were pre-treated with Abl kinase inhibitor imatinib (1-100 μM) or nilotinib (1-100 μM) for 30 min at 37 °C, respectively. In the presence of inhibitors, cells were incubated with *P. aeruginosa* (MOI of 100) for 2 h at 37 °C. Cells were washed three times with Dulbecco's phosphate-buffered saline (DPBS). Extracellular bacteria were inactivated by a 2 h treatment of cells at 37 °C with 400 μg·ml⁻¹ Amikacin sulfate. Subsequently, cells were washed two times with DPBS and lysed with 0.25 % (vol/vol) Triton X-100 at 37 °C. Cell extracts were plated on Gentamicin-containing (60 μg·ml⁻¹) LB-Miller Agar plates and incubated over night at 37 °C. The next day bacterial colonies were counted. Invasion was calculated as percentage of Amikacin-survived bacteria compared with the total number of bacteria associated with Amikacin-untreated cells. For comparison purposes, mean values of $n \geq 3$ independent experiments were normalized to the invasion into inhibitor-untreated H1299 cells.

4.4 Lectin stimulation, RNA interference, SDS-PAGE and immunoblot analysis

LecA was expressed and purified from *E. coli* BL21 (DE3) transformed with the LecA encoding plasmid pET25-pa11 as described earlier [28]. Labelling of purified LecA with the fluorophore Alexa488 (Thermo Fisher Scientific) was performed according to the manufacturer's protocol. Lyophilized, recombinant LecA was dissolved in DPBS (supplemented with Ca²⁺, Mg²⁺), and used at a concentration of 100 nM. Cells were seeded to reach 70-80 % confluency, starved for 2 h and stimulated as indicated. Starvation was performed to exclude possible effects of growth factors and cytokines inherently present in FCS, which might interfere with LecA effects. Cells were harvested and lysed in modified radio-immunoprecipitation assay (RIPA) buffer (20 mM Tris (pH 8.0), 0.5 % (w/vol) Na-deoxycholate, 13.7 mM NaCl, 10 % (vol/vol) Glycerol, 0.1 % (w/vol) SDS, 2 mM EDTA, 200 μM Pefabloc, 11 μM Leupeptin, 0.8 μM Aprotinin, 1 mM Na-orthovanadate, 1 % (vol/vol) Phosphatase Inhibitor Cocktail 3 for at least 45 min at 4 °C. Cell lysates were collected and centrifuged to remove cell debris. Knock down of Abl was performed by RNA interference (RNAi). H1299 cells were transfected with 80-100 pmol of scrambled or Abl-specific siRNA (Santa Cruz, Cat. No. sc-37007 and sc-29843) using Lipofectamine (Thermo Fisher Scientific). Knock down efficiency was about 80 % as quantified by densitometric analysis of Abl Western blots.

The protein concentration of each extract was determined by using the Pierce BCA Protein Assay Kit. Equal amounts of proteins were loaded and separated on 8 % Tris-glycine SDS-PAGE gels and then transferred on nitrocellulose membranes. Membranes were blocked in 3 % BSA prepared with TBST (50 mM Tris (pH 7.6), 154 mM NaCl, 0.5 % (vol/vol) Tween 20) and incubated with primary antibodies (1:1000) at 4 °C overnight. Next day, horseradish peroxidase conjugates appropriate for each IgG (1:2000) were used to detect primary antibodies. Blot stripping was performed if necessary. ClarityTM Western ECL Chemiluminescent Substrate was used for detection of bound peroxidase. Luminescence was detected using Vilber Lourmat Fusion FX chemiluminescence imager. α-Tubulin was used as a loading control in all immunoblot experiments. If not indicated otherwise, α-Tubulin normalization of phospho-CrkII was justified by proofed correlation with Crk normalization (Suppl. Fig. 5). For comparison purposes, mean values of CrkII phosphorylation of $n \geq 3$ independent experiments were normalized to uninfected or untreated H1299 and CHO cells, respectively.

4.5 Immunofluorescence microscopy and FACS

H1299 cells were seeded on glass coverslips. The next day cells (70-80 % confluent) were starved for 2 h at 37 °C with FCS-free medium. Starvation was performed to achieve results that are comparable to Western blot experiments. Meanwhile, LecA (100 nM), which was labeled with Alexa 488, was incubated with PNPG (0.1-5 mM) for 15 min at 37 °C. Cells were incubated with LecA-Alexa488 (with or without PNPG) for 60 min at 37 °C. Afterwards, cells were fixed with 4 % paraformaldehyde, saturated with 50 mM NH₄Cl and permeabilized with a buffer containing 0.2 % (w/vol) BSA, 0.02 % saponin (w/vol) in DPBS for 20 min at room temperature. Finally, cells were counterstained with Phalloidin-ATTO647N (1:2000), DAPI (1:5000) and mounted with Mowiol containing DABCO. Samples were imaged on a confocal microscope (Nikon Eclipse Ti-E with Nikon A1R confocal laser scanner, 60 × oil objective, N.A. 1.49). Image acquisition and analysis were performed with NIS-Elements (Nikon). For FACS analysis, H1299 cells were treated with LecA-Alexa488 (with or without PNPG) as described above, then scraped and re-suspended in DPBS.

4.6 Antibodies and chemical reagents

The following antibodies were obtained from commercial sources: polyclonal rabbit anti-Crk p38 (phospho Y221) (Abcam, Cat. No. ab51222, RRID:AB_869267), monoclonal mouse anti-Crk (BD Biosciences, Cat. No. 610035, RRID:AB_397451), monoclonal rabbit anti-Src Phospho (pY418) (Abcam, Cat. No. 5595-1, RRID:AB_10999890), monoclonal rabbit anti-Src (32G6) mAb (Cell Signaling Technology, Cat. No. 2123, RRID:AB_10695000), polyclonal rabbit anti-c-Abl (Cell Signaling Technology, Cat. No. 2862, RRID:AB_2257757), monoclonal mouse anti-β-Actin (Sigma-Aldrich, Cat. No. A5316, RRID:AB_476743), monoclonal mouse anti-α-Tubulin (Sigma-Aldrich, Cat. No. T5168, RRID:AB_477579), horse anti-mouse-HRP (Cell Signaling Technology, Cat. No. 7076, RRID:AB_330924) and goat anti-rabbit-HRP (Cell Signaling Technology, Cat. No. 7074, RRID:AB_2099233). LecA was detected by a polyclonal rabbit anti-LecA antibody. Pierce BCA Protein Assay Kit and Clarity™ Western ECL Chemiluminescent Substrate were purchased from Thermo Scientific and BIO-RAD, respectively. Abl kinase inhibitor imatinib (mesylate) was obtained from Biovision, other inhibitors nilotinib, dasatinib and GNF-2 were from Santa Cruz. Purified DL-PPMP and PNPG were obtained from Santa Cruz and Sigma-Aldrich, respectively. SFK inhibitor KB SRC4 was from TOCRIS bioscience. The proteases Aprotinin, Leupeptin, Pefablock, Sodium orthovanadate and Phosphatase Inhibitor Cocktail 3 were all purchased from Sigma-Aldrich. RPMI 1640 and DMEM, DPBS, FCS and L-Glutamine were all purchased from Gibco. The following chemicals were obtained from Roth: BSA, DABCO, DAPI, EDTA, Geneticin, Glycerol, LB, Mowiol, NaCl, Na-deoxycholate, NH₄Cl, paraformaldehyde, SDS, Tris (hydroxymethyl)-aminoethane, Triton X-100 and Tween 20. Amikacin sulfate, Gentamicin, Phalloidin-ATTO647N and Saponin were obtained from Sigma-Aldrich. StxB was purchased from Sigma-Aldrich (Cat. No. SML0562).

4.7 Statistical analysis

All data are presented as mean ± standard deviation (SD) values that have been calculated from the results of independent experiments. Statistical testing was performed with Microsoft Excel (Microsoft Office Standard 2010) and GraphPad Prism software (5.04) with data of $n \geq 3$ independent experiments. Differences to the controls were analysed for significance by using one-way analysis of variance (ANOVA), with a Dunnett's Multiple Comparison Test compared to the control conditions.

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Figure

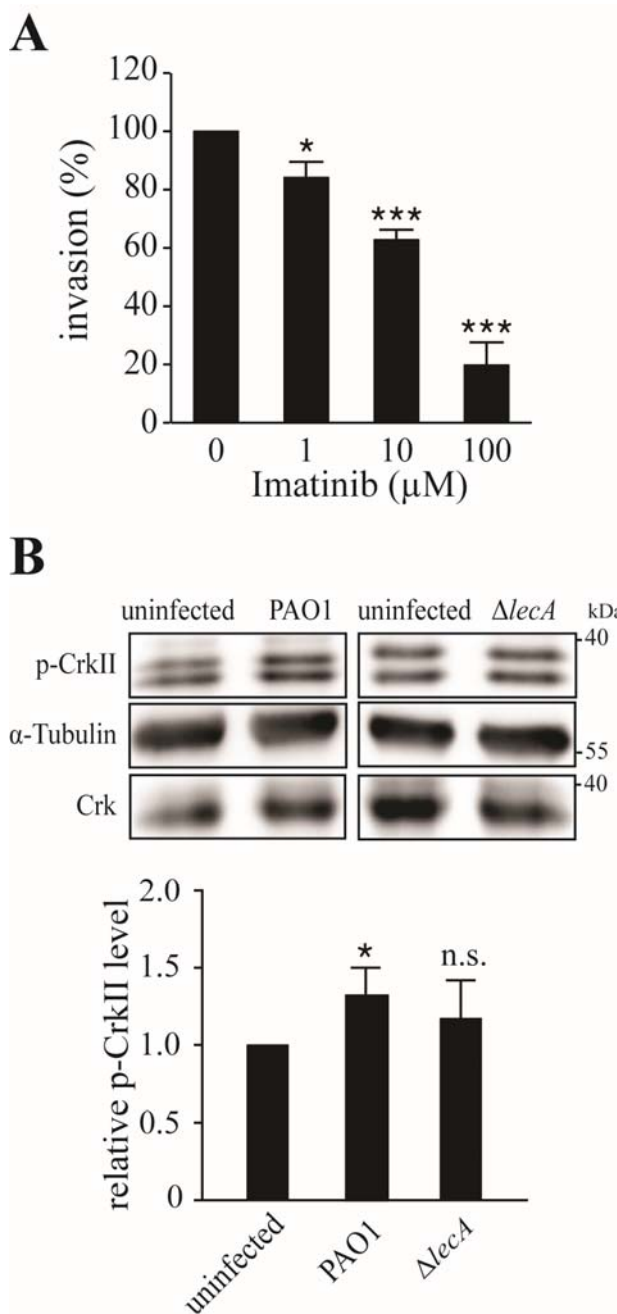


Fig. 1: *P. aeruginosa* infection of human lung epithelial cells depends on tyrosine kinases and leads to phosphorylation of the adaptor protein CrkII. (A) *P. aeruginosa* invasion is dependent on tyrosine kinase activity. H1299 cells were infected with GFP-tagged PAO1 for 2 h in the presence of the tyrosine kinase inhibitor imatinib (0-100 μM), and bacterial invasion was quantified. The results were normalized with respect to invasion in untreated cells. One-way ANOVA, Dunnett’s Multiple Comparison Test, * $p < 0.05$, *** $p < 0.001$ compared to uninfected cells. (B) *P. aeruginosa* induces tyrosine phosphorylation of CrkII in human lung epithelial cells. H1299 cells were infected for 60 min with wild type PAO1 or a LecA deletion mutant of PAO1 ($\Delta lecA$). Total cell lysates were immunoblotted with indicated antibodies (left). Phospho-CrkII^{Y221} levels were quantified relative to α -Tubulin and normalized with respect to uninfected cells (right). Student’s t-test, n.s.: non-significant compared to uninfected cells, * $p < 0.05$ compared to uninfected cells. Error bars show SD; n=3 biological experiments.

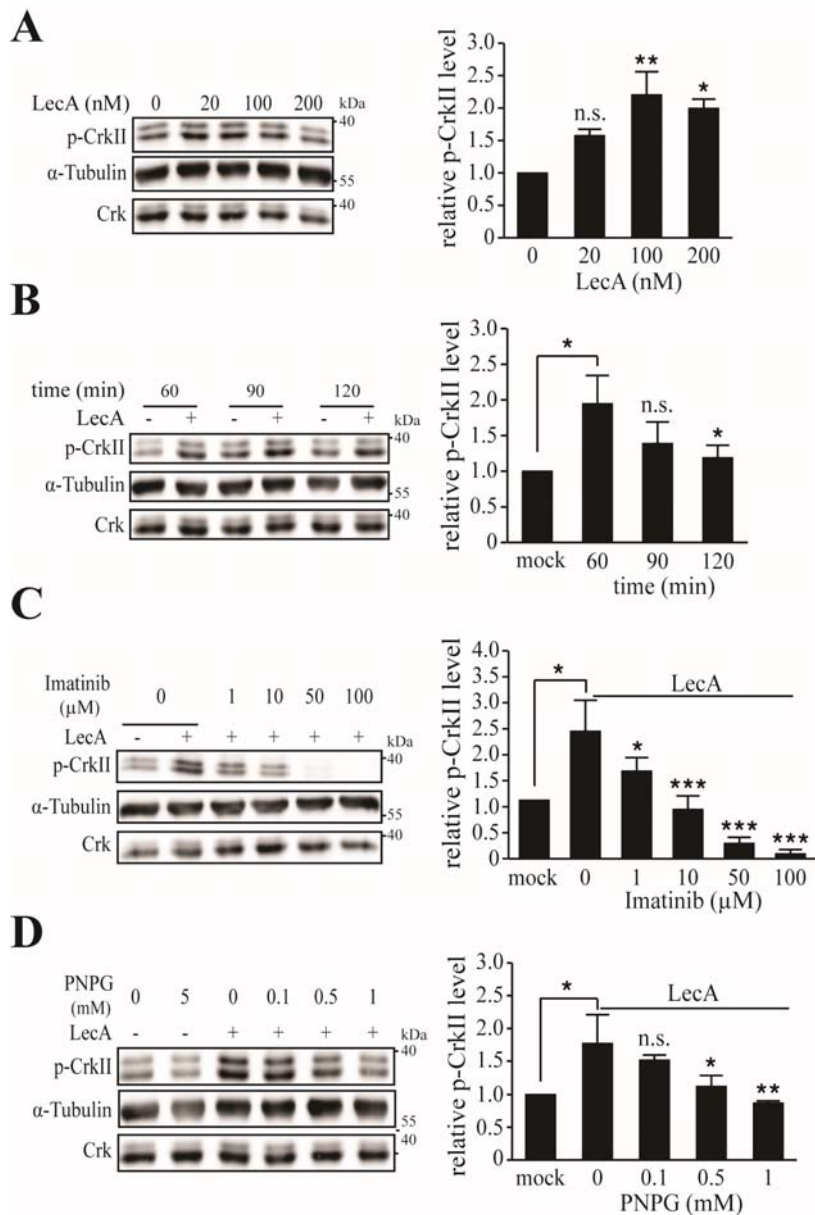


Fig. 2: *P. aeruginosa* lectin LecA triggers CrkII^{Y221} phosphorylation in human lung epithelial cells. (A) LecA induces CrkII^{Y221} phosphorylation in a dose-dependent manner. (left) H1299 cells were incubated for 60 min in the presence of LecA (0-200 nM). (right) Phospho-CrkII^{Y221} levels were normalized with respect to untreated cells. (B) CrkII is transiently phosphorylated by LecA. (left) H1299 cells were incubated for 60 min in the presence of LecA (100 nM), then LecA was removed and cells were further incubated for 30 min and 60 min, respectively. (right) Phospho-CrkII^{Y221} levels were normalized with respect to untreated cells, at the corresponding time points. (C) Phosphorylation of CrkII^{Y221} is sensitive to imatinib (left) H1299 cells were stimulated for 60 min in the presence of imatinib (0-100 μ M) and LecA (100 nM). Imatinib was pre-incubated with cells for 30 min at 37 °C and maintained during stimulation. (right) Phospho-CrkII^{Y221} levels were normalized with respect to DMSO-treated cells (mock). (D) Inhibition of LecA results in decreased CrkII^{Y221} phosphorylation. (left) H1299 cells were stimulated for 60 min in the presence of PNPG (0-1 mM) and LecA (100 nM) or PNPG (5 mM) only. LecA was pre-incubated with PNPG for 15 min at 37 °C and maintained during stimulation. (right) Phospho-CrkII^{Y221} levels were normalized with respect to PNPG-only-treated cells. Total cell lysates were immunoblotted with indicated antibodies. Phospho-CrkII^{Y221} levels were quantified relative to α -Tubulin. Error bars show SD; n=3 biological experiments. One-way ANOVA and Dunnett's Multiple Comparison Tests were performed. In figures B, C and D, mock samples were compared to 60 min (B), imatinib (0 μ M, C) and PNPG (0 mM, D), respectively, by Student's t-test. n.s.: non-significant compared to mock samples. * p<0.05; ** p<0.01; *** p<0.001.

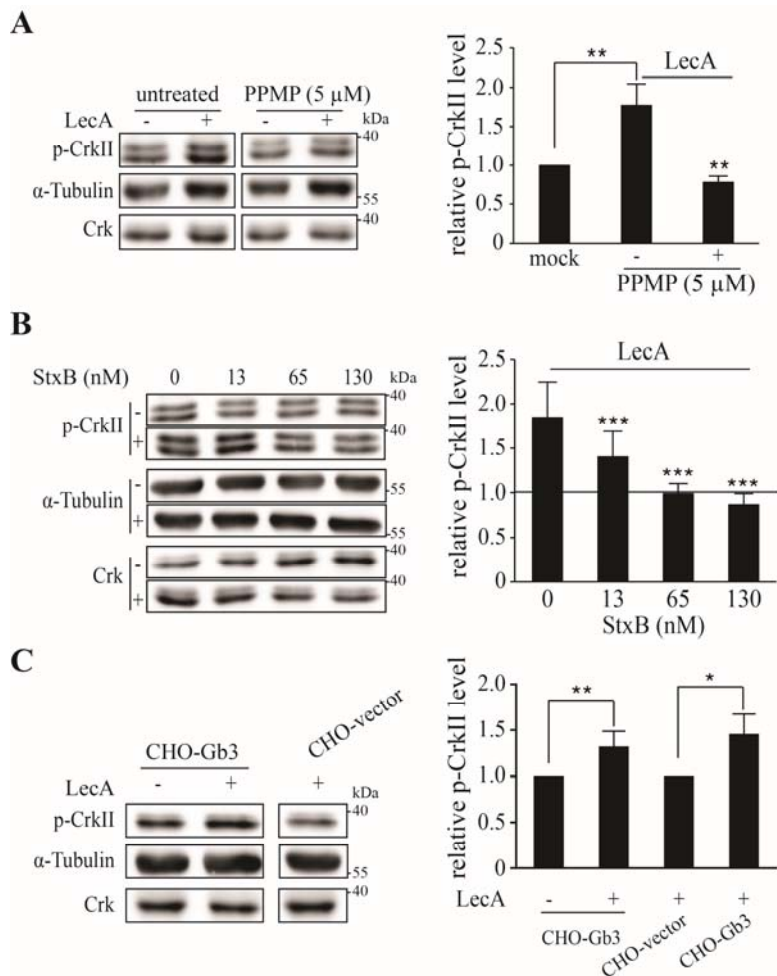


Fig. 3: Glycosphingolipid Gb3 is crucial for LecA-induced CrkII^{Y221} phosphorylation in mammalian cells. (A) GSLs mediate LecA-dependent Abl kinase activation. (left) H1299 cells, pre-treated without or with DL-PPMP (5 μM), were stimulated for 60 min in the presence of LecA (100 nM). (right) Phospho-CrkII^{Y221} levels were normalized with respect to untreated cells. (B) Gb3 is crucial for LecA-induced Abl kinase activation. (left) H1299 cells were stimulated with StxB (0-130 nM) for 30 min, then StxB was removed, and cells were further incubated with (+) or without (-) LecA (100 nM) for 60 min. (right) Phospho-CrkII^{Y221} levels were normalized with respect to only StxB-treated cells. The horizontal line indicates the basal level of phospho-CrkII^{Y221} after StxB treatment. (C) Gb3 in particular is required for LecA-induced Abl kinase activation in CHO transfected cells. (left) CHO cells stably transfected with Gb3 synthase (CHO-Gb3) or empty vector (CHO-vector) were stimulated with LecA (100 nM) for 60 min. (right) Phospho-CrkII^{Y221} levels were normalized with respect to empty vector-transfected CHO cells treated with LecA. Total cell lysates were immunoblotted with indicated antibodies. Phospho-CrkII^{Y221} levels were quantified relative to α-Tubulin. Error bars show SD; n=3 biological experiments. One-way ANOVA and Dunnett's Multiple Comparison Test were performed. In figure A, the mock sample was compared to DL-PPMP (5 μM) (-), in figure C, the CHO-Gb3 sample was compared to the CHO-vector sample, respectively, by Student's t-test. * p<0.05; ** p<0.01; *** p<0.001.

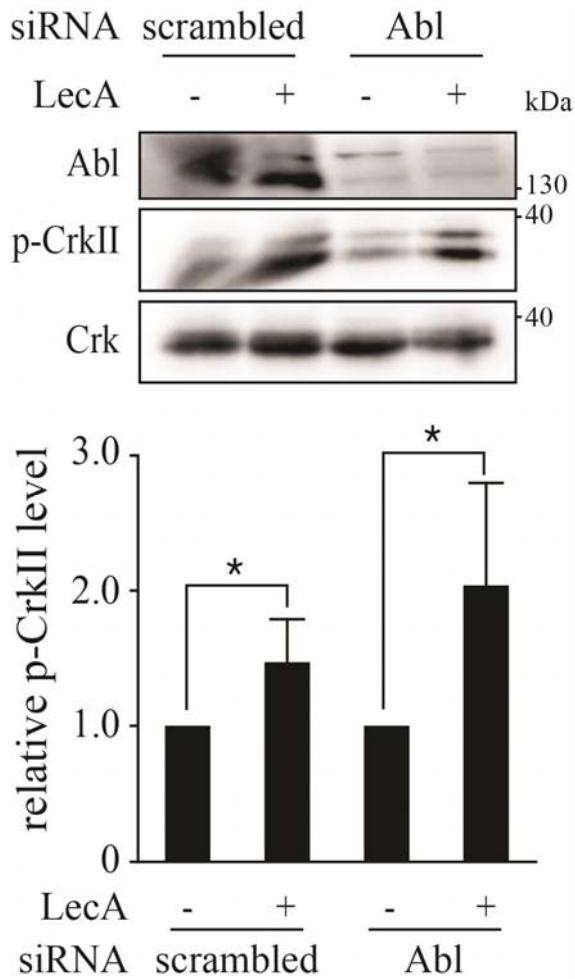


Fig. 4: Abl kinase is not involved in LecA-induced CrkII^{Y221} phosphorylation in human lung epithelial cells

H1299 cells were transfected for 2-3 days with 80-100 pmol of Abl-specific or scrambled siRNA and thereafter incubated with 100 nM LecA for 60 min at 37 °C. Total cell lysates were immunoblotted with indicated antibodies. Phospho-CrkII^{Y221} levels were quantified relative to Crk. Error bars show SD; n=5 biological experiments. Untreated samples were compared LecA-treated samples, respectively, by Student's t-test. * p<0.05.

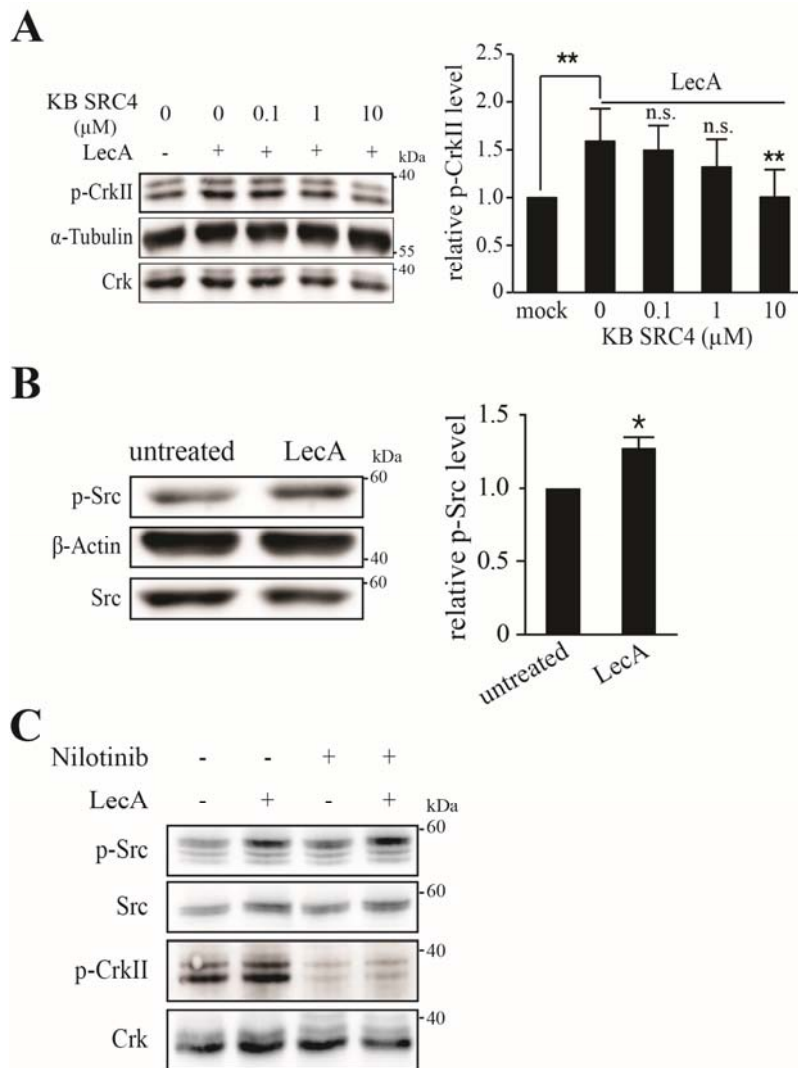


Fig. 5: Src family kinases mediate LecA-stimulated CrkII^{Y221} phosphorylation in human lung epithelial cells. (A) H1299 lung epithelial cells were pre-incubated with KB SRC4 (0-10 μM) for 30 min at 37 $^{\circ}\text{C}$, then, cells were stimulated with LecA (100 nM) for 60 min in the presence of KB SRC4. Total cell lysates were immunoblotted with indicated antibodies. Phospho-CrkII^{Y221} levels were quantified relative to α -Tubulin and normalized with respect to DMSO-treated cells (mock). Error bars show SD; n=3 biological experiments. One-way ANOVA and Dunnett's Multiple Comparison Test were performed. The mock sample was compared to the KB SRC4 sample (0 μM) by Student's t-test. n.s.: non-significant compared to mock sample. ** $p < 0.01$. (B) H1299 cells were incubated for 60 min in the presence of LecA (100 nM). Total cell lysates were immunoblotted with indicated antibodies. Phospho-Src levels were quantified relative to β -Actin. Phospho-Src^{Y418} levels were normalized with respect to untreated cells. Error bars show SD; n=3 biological experiments. Untreated samples were compared to LecA-stimulated samples by Student's t-test. * $p < 0.05$. (C) H1299 cells were pre-treated with Nilotinib (1 μM) and subsequently incubated with LecA (100 nM) for 60 min at 37 $^{\circ}\text{C}$. Total cell lysates were immunoblotted with indicated antibodies.