รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาการทำหน้าที่ของโปรตีน Nck ที่สัมพันธ์กับการทำงานของเอนไซม์ Lck ในการกระตุ้น T lymphocytes

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยนเรศวร
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว และมหาวิทยาลัยนเรศวรไม่จำเป็นต้องเห็นด้วยเสมอไป)

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กิตติกรรมประกาศ

โครงการวิจัยนี้ได้รับทุนจากสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) และทุนบางส่วนจากมหาวิทยาลัยนเรศวรให้การดำเนินการวิจัยมีประสิทธิภาพ และผู้วิจัยได้รับการสนับสนุนเป็นอย่างดียิ่งจากคณะแพทยศาสตร์ ในด้านห้องปฏิบัติการวิจัยและเวลาในการทำวิจัยตลอดมา ผู้วิจัยขอขอบพระคุณ ศาสตราจารย์เกียรติคุณ.ดร.สพ.ญ.วันเพ็ญ ชัยคำภา ศาสตรจารย์ ดร.นายแพทย์อภิวัฒน์ มุทิรางกูร ศาสตราจารย์เกียรติคุณ ดร.ประพนธ์ วิไลรัตน์ และเมธีวิจัยอาวุโสสาขาวิทยาศาสตร์การแพทย์อีกหลายท่านที่ผู้วิจัยไม่ได้เอ่ยนามในที่นี้ ที่ได้กรุณาให้คำแนะนำและกำลังใจในการทำวิจัยที่มีคุณค่ายิ่งตลอดมา นอกจากนี้ ขอขอบคุณผู้บริหารคณะกรรมการและเจ้าหน้าที่ของ สกว. ทุกท่านที่เอื้อเฟื้อ สนับสนุน และอำนวยความสะดวกในการทำงานด้านวิชาการและการประชุมสัมมนาต่าง ๆ เป็นอย่างดียิ่ง

Abstract

T lymphocyte or T cell specifically recognizes antigenic peptide associated with major histocompatibility complex (MHC) molecules, by using T cell receptor-CD3 complex (TCR-CD3). This present work investigated the function of an adaptor protein Nck in relation to the function of an enzyme kinase Lck, which is directly involved in the TCR/CD3 proximal activation upon binding with peptide-MHC molecule. This was emphasized on the binding of the two proteins in terms of the conformation of Lck enzyme as well as the phosphorylated site on the Nck (non-catalytic region of tyrosine kinase) molecules. In this present work, the site-directed mutagenesis of Lck gene was performed to obtaine various conformations of Lck proteins expressed in JCam 1.6, a model of T cells that lacks Lck enzyme. In addition, mutant Nck at tyrosine 105 was constructed to study the phosphorylation of this residue associated with the function of Lck kinase. Work on Lck mutants expressed in J Cam 1.6 T cells would shed some light on understanding about Nck-Lck interaction in activated T cells. Lck was found to be important in CD3-Nck binding. I have reported in Journal of Immunology about the role of Nck in T cell activation with the novel findings that the Nck has a specific pattern of binding to CD3 epsilon chain which has led to a review issue in Immunology about the importance of signaling molecules including Nck adaptor protein in T cell activation. The knowledge of TCR proximal activation also led to another manuscript under revision for Frontiers in Immunology. The results obtained will shed light on molecular interaction of Nck-CD3 epsilon and Lck enzyme in T cell signaling and function of T cells.

บทคัดย่อ

T cell สามารถจดจำและจับ antigen peptide ที่อยู่กับโมเลกุล major histocompatibility complex (MHC) ได้อย่างจำเพาะโดยการใช้ T cell receptor-CD3 (TCR-CD3) complex โครงการวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาการทำหน้าที่ของโปรตีน Nck ที่เกี่ยวข้องกับการทำงานของเอนไซม์ Lck ซึ่งเกี่ยวข้องโดยตรงในการกระตุ้น T cell ที่จับกับ peptide-MHC โดยเน้นที่การจับกันของโปรตีนและเอนไซม์ดังกล่าวว่ามีบทบาทหรือไม่อย่างไรในการกระตุ้น T cell ขั้นตอนการวิจัยคือทำการทดลองว่า conformation ของเอนไซม์ Lck เกี่ยวข้องอย่างไรกับการจับกับโปรตีน Nck เมื่อมีการกระตุ้น T lymphocyte ผ่านทาง T cell receptor ทำ site directed mutagenesis ของยีน Lck เพื่อให้ได้ยีนที่มีลักษณะของโปรตีนแบบต่าง ๆ ที่ต้องการแล้วจึงนำยีนเหล่านี้ transfect เข้าไปใน T cell ในที่นี้ใช้เซลล์ Jurkat ชนิด JCam 1.6 ซึ่งเป็นเซลล์ที่ขาดเอนไซม์ Lck จากนั้นจะนำเซลล์ที่ได้มาทดลองกระตุ้นผ่าน T cell receptor เพื่อศึกษาผลของการกระตุ้นที่นำไปสู่การจับกันของโมเลกุลที่เกี่ยวข้องโดยตรงในกระบวนการส่งสัญญาณเข้าสู่นิวเคลียสไปตามลำดับ ขณะเดียวกันได้ทดลองเกี่ยวกับการที่โมเลกุล Nck จะถูกเติมหมู่ฟอสเฟตได้โดย Lck ขณะนี้ได้เซลล์ที่มีโมเลกุล Nck ที่มีการกลายพันธุ์ของตำแหน่งไทโรซีนที่ 105 ซึ่งคาดว่าจะเกี่ยวข้องกับ phosphorylation โดยเอนไซม์ Lck เพื่อเป็นพื้นฐานความเข้าใจและในการทดลองเชิงลึกต่อไปเกี่ยวกับบทบาทของโมเลกุลนี้ในการทำหน้าที่ของ T cell ผลการทดลองพบว่าเอนไซม์ Lck มีความเกี่ยวข้องกับการจับกันของ Nck และ CD3 epsilon ซึ่งพบว่ามีแบบแผนการจับกันที่มีลักษณะเฉพาะ ทำให้ผู้วิจัยได้เผยแพร่ผลการวิจัยในวารสาร Journal of Immunology ในปีที่ผ่านมา และงานสังเคราะห์ความรู้ในวารสาร Immunology ในปีนี้ ขณะเดียวกันความรู้จากการทำปฏิกิริยาในตอนต้นของ TCR ทำให้ได้มีงานวิจัยในวารสาร Frontiers Immunology ซึ่งขณะนี้อยู่ในระหว่างปรับเพิ่มเติมผลการทดลองบางส่วนด้วย

Executive Summary

เนื้อหางานวิจัย

The T cell receptor (TCR) is associated with CD3 complex molecules (TCR/CD3). A T cell generally recognizes an antigenic peptide presented by an MHC molecule (pMHC molecule) on the surface of an antigen presenting cell (APC). Apropriate binding affinity of TCR and pMHC induces an enzyme called Lck (lymphocyte-specific protein tyrosine kinase) to phosphorylate the ITAMs (immune receptor tyrosine-based activation motifs) at the cytoplasmic tali of the TCR-CD3 complex. Then another enzyme known as ZAP-70 kinase is recruited to bind the phosphorylated ITAMs leading to phosphorylation and recruitment of other enzymes and adaptor protein molecules. These include, for instance, PLCγ (phospholipase Cγ), LAT (linker for activation of T cells), SLP-76 (SH2-domain-containing leukocyte protein of 76 kDa), and Nck (non-catalytic region of tyrosine kinase) [Samelson, 2002] to form a signaling complex that is important for activation of many signaling pathways and T cell activation [Horejsi *et al*., 2004; Koretzky *et al*., 2006]. Activation of T cells lead to T cell proliferation and differentiation as well as production of various cytokines and growth factors [Choudhuri *et al*., 2005; Smith-Garvin *et al*., 2009].

Nck is an adaptor protein composed of three SH3 domains at the amino-terminal and one SH2 domain at the carboxy-terminal [Buday *et al*., 2002; Lettau *et al*., 2009]. In humans, there are 2 Nck isoforms that are Nck1 or Nckα and Nck2 or Nckβ (or another name is Grb4). The main function of Nck is to interact with other proteins to form a signaling complex. Studies in several cell types have shown that Nck can interact with more than 60 types of protein [Buday *et al*., 2002; Li *et al*., 2001]. Nck binds to phosphorylated protein via its SH2 domain and binds to proline-rich sequence via its SH3 domain. Thus, Nck functions to link a signal from the upstream phosphotyrosine (pTyr) via SH2 domain to the downstream effector via SH3 domain [Lettau *et al*., 2009].

Lck is a kinase enzyme that plays a key role in T cell activation [Broman et al., 2004; Parsons and Parsons, 2004]. Upon TCR stimulation by binding with pMHC, the Lck molecules phosphorylate ITAMs of the TCR-CD3 complex to initiate intracellular signaling. Lck molecule is made up of lipids that undergo lipid modification including myristolylation and palmitoylation at the N-terminal. This structure is used to bind cell membranes. The N-terminal of Lck also contains 2 cysteine molecules for binding with CD4 or CD8 molecule. Lck has one SH3 and one SH2 domain, which can bind other proteins. Its SH3 domain binds to proline-rich sequence while its SH2 domain binds to phosphorylated protein. In addition, it is noted that the amino acids located between lipid modification and the SH3 domain are also proline-rich sequence that could bind other protein that carries SH3 domain. The C-terminal of Lck molecule is a catalytic tyrosine kinase domain [Turner et al., 1990; Kim et al., 2003].

Activation of Lck is induced by binding of TCR to pMHC. It is thought that activation of Lck molecules are associated with binding of CD4 or CD8 co-receptor molecules to MHC class II or MHC class I molecules, respectively. This is because Lck molecules are found to be associated with the CD4 and CD8 molecules on the cell membranes [Rudd et al., 1998, Veillette et al., 1988]. Moreover, activation of double

positive thymocytes is involved in simultaneous stimulation of TCR and co-receptors [Wiest et al., 1996]. However, deletion of CD4 or CD8 molecules does not affect T cell development and activation [Van Laethem et al., 2007]. Recent studies have shown that there are 2 types of Lck molecules. One is membreane associated with CD4 and CD8 molecules and the other one is free in the cytoplasm. This may explain the dispensable role of CD4 and CD8 molecules in thymocyte development as some Lck molecules exist in the cytoplasm. It has also demonstrated that up to 40% of Lck molecules are in a stimulatory state even though the T cell is not activated and stimulation of TCR or co-receptors does not increase the activity of Lck [Turner JM, 1990; Nika et al., 2010]. In many studies, for stimulation of primary T cells in vitro, anti-CD3 and anti-CD28 antibodies are generally used that can stimulate Lck, ITAMs and other proteins leading to T cell activation. Thus, Lck molecules may be activated without any involvement of co-receptors CD4 and CD8 molecules. The distribution of activated Lck molecules are found to be clustered with phosphorylated TCR molecules. Therefore, stimulation of TCR is believed to recruit Lck to the TCR-CD3 complex [Rossy et al., 2013].

After TCR ligation, there is a conformational change at the CD3ε causing an exposure of the proline-rich sequence (PRS) of the cytoplasmic tail of the CD3ε. Then, Nck molecules are recruited to bind the CD3ε. The Nck uses its SH3.1 domain to bind the PRS of the CD3ε. This is thought to be important for stimulation of other proteins essential for phosphorylation of ITAMs leading to T cell activation [Gil et al., 2002]. As aforementioned that Lck also contains the PRS that can bind the Nck, it is possible that the recruitment of Lck to the TCR may relie on Nck molecules functioning as a mediator. When TCR is activated, binding of Nck to the PRS of the CD3ε may facilitate the recruitment of Lck molecules to the CD3ε. Lck molecules then phosphorylate ITAMs of the TCR-CD3 complex, thus, initiating the intracellular signaling and activation of the T cell. Nevertherless, there has been no direct evidence that shows that stimulation of Lck is a direct result of TCR activation. It is also questioned that whether or not the Nck is directly involed in Lck stimulation. Preliminary studies in my lab showed that when Jurkat T cells were stimulated with anti-TCR antibody, the Lck molecules were co-precipitated with CD3 molecules. In Nck1 and Nck2 knock-down Jurkat cells, there was a decline in co-precipitation of Lck and CD3 molecules. This finding demonstrates that upon T cell activation, Lck molecules are recruited to the TCR-CD3 complex and the Nck1 and Nck2 may function as a mediator. Besides that finding, Lck was also co-precipitated with Nck1 and Nck2. From these data, this study investigated activation of the TCR involving a recruitment of cytoplasmic Lck molecules to the TCR-CD3 complex and Nck adaptor protein. This event would be very important for the initation of T cell activation.

From the work we have performed experiments and reported about the pattern of Nck binding to the CD3 epsilon which comprises the SH3 domain of Nck binding to the PRS of CD3 and the SH2 Nck binding to the partial phosphorylated CD3 in Journal of Immunology. We also hypothesized and synthesis the signaling molecule mechanisms in a review issue of Immunology. In addition with a collaboration with Prof. Wolfgang Schamel in Freiburg University, Germany, we are reporting the differential function of Nck in the so called gamma/delta T cells in killing tumor cells in vitro and this manuscript is under minor revision for Frontiers in

Immunology at the time of writing this report. Work is also carried forward the study the role of Lck-Nck interaction in proximal TCR/CD3 proximal signaling.

The methods that were used in the present work are as followed

1) site-directed mutatgenesis of Lck gene: In order to construct the plasmids containing genes of various Lck mutants, including Lck (Y505F), Lck (Y394F), and Lck (Y505F,K293R), the plasmids that contain wild-type Lck will be used as a template. This is kindly provided by Prof. Dr Katharina Gaus, University of New South Wales, Sydney, Australia. Gene mutation and polymerase chain reaction (PCR) will be done using QuickChangeII Site-Directed Mutatgenesis kit (Stratagene) according to the manufacturer's instructions and as previously reported by our group and others [Ngoenkam et al 2014; Bondzi et al., 2000; Derkinderen P et al., 2005; Levin SD. et al., 1993; Nakahira K et al., 2013;]. After performing the PCR, *Dpn I* is added into the PCR tube and it is mixed by pipetting up and down before it is spun down. Then the sample is incubated at 37°C for 1 hour to digest the parental plasmid. After that, the *Dpn I*-treated plasmid DNA is transformed into XL1-Blue supercompetent cells before the cells are spread on LB agar plate containing ampicillin antibiotic that is then incubated at 37°C for 16 hours. The bacterial colony that grows on the agar plate is then taken to culture for next plasmid extraction. The precise order of nucleotides is then examined by DNA sequencing.

2) Gene transfection: Gene transfection will be performed to transfect wild-type (WT) and mutant Lck genes into the Lck-deficient Jurkat T cells (J.CaM1). Briefly, JCaM1 cells are cultured in complete RPMI 1640 medium. The cells are counted for electroporation using Neon™ Transfection system (Invitrogen) with the plasmids added at 1 microgram/ 1 reaction. The desired plasmids are WT Lck-GFP, Lck10-GFP, Lck (Y505F), Lck (Y394F), Lck (Y505F,K273R), Lck (C20/23A), and Lck (PRS mutant). The transfection assay is performed according to the kit's instruction with the pulse voltage/pulse width/pulse number of 1600/10/3 for microporation using the Digital Bio Technology, Taiwan. The transfected cells are then cultured for another 48 hours before they are lysed in lysis buffer and protein expression is then examined using protein detection kit (Peirce) according the kit's instruction. The expression of the Lck is studied using immunoblotting with antibodies against Lck and beta-actin for loading control.

3) Immunoprecipitation: Immunoprecipitation of the reconstituted JCaM1 cells will be performed after the cells are stimulated with anti-TCR (C305) IgM antibody at different time points. The objective is to precipitate the proteins of interest including CD3ε (being bound with Nck to examine for CD3ε-Nck interaction) and Nck (being bound with Lck to examine for Nck-Lck interaction). The total cell lysates are prepared using lysis buffer specially prepared for immunoprecipitation as previously reported by my research group [Yiemwattana et al., 2013, Ngoenkam et al., 2014]. The immunoprecipitation is performed using protein

G-Sepharose beads together with specific antibody for the protein of interest. The precipitated products are studied using SDS-PAGE using 10% polyacrylamide gel as separating gel for futher immunoblotting.

4) Immunoblotting: Immunoblotting, or Western blotting, will be used to detect the protein of interest using specific antibody. In this work the band of protein will be analyzed by an enhanced chemiluminescence method.

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IMMUNOLOGY REVIEW ARTICLE

Selected signalling proteins recruited to the T-cell receptor–CD3 complex

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Summary

The T-cell receptor (TCR)–CD3 complex, expressed on T cells, determines the outcome of a T-cell response. It consists of the TCR-αβ heterodimer and the non-covalently associated signalling dimers of CD3εγ, CD3εδ and CD3ζζ. TCR-αβ binds specifically to a cognate peptide antigen bound to an MHC molecule, whereas the CD3 subunits transmit the signal into the cytosol to activate signalling events. Recruitment of proteins to specialized localizations is one mechanism to regulate activation and termination of signalling. In the last 25 years a large number of signalling molecules recruited to the TCR–CD3 complex upon antigen binding to TCR-αβ have been described. Here, we review knowledge about five of those interaction partners: Lck, ZAP-70, Nck, WASP and Numb. Some of these proteins have been targeted in the development of immunomodulatory drugs aiming to treat patients with autoimmune diseases and organ transplants.

Keywords: protein–protein interaction; signal transduction; T-cell activation; T-cell receptor–CD3 complex.

Introduction

Immune responses to infectious pathogens serve to maintain body homeostasis. Among various immune cells, T cells play an important role to fulfil this critical function. A T-cell response to foreign antigen is initiated by the binding of the T-cell receptor (TCR)–CD3 complex to a foreign peptide bound to an MHC molecule presented on an antigen-presenting cell. Information of this binding is transmitted into the cytosol to activate many signalling proteins.[1,2] The final targets are transcription factors, to alter the gene expression profile, metabolic enzymes, to change metabolic activity,[3] and cytoskeletal rearrangement. Together this leads to cell proliferation and effector

Abbreviations: CAR, chimeric antigen receptor; ERK, extracellular signal-regulated kinase; ITAMs, immunoreceptor tyrosine-based activation motifs; LAT, linker for the activation of T cells; Lck, lymphocyte-specific protein tyrosine kinase; Nck, non-catalytic region of tyrosine kinase; PRS, proline-rich sequence; SH, Src-homology; SLP-76, SH2-domain-containing leucocyte protein of 76 000 MW; TCR, T-cell receptor; TSAd, T-cell specific adaptor protein; VCA, verprolin homology domain-cofilin homology domain-acidic region; WASP, Wiskott–Aldrich syndrome protein; WAS, Wiskott–Aldrich syndrome; ZAP-70, ζ chain-associated protein kinase of 70 000 MW

molecule production and secretion, which are crucial for T-cell-mediated immune responses.[4]

The TCR–CD3 complex is a multisubunit protein complex. It is composed of an antigen-binding TCRαβ heterodimer non-covalently associated with the non-variable signal transduction subunits; the CD3 heterodimers CD3$\varepsilon\gamma$ and CD3$\varepsilon\delta$ as well as the CD3$\zeta\zeta$ homodimers.[5–7] The cytoplasmic tails of CD3ε, CDδ, and CD3γ each contain one immunoreceptor tyrosine-based activation motif (ITAM) and that of CD3ζ contains three ITAMs, hence one TCR–CD3 complex comprises 10 ITAMs. The conserved amino acid sequence of the ITAMs is D/ExYxxLx (6–8)YxxL. Antigen binding to TCR-$\alpha\beta$ results in phosphorylation of the ITAM residues, leading to recruitment and activation of multiple downstream signalling molecules including enzymes and adaptor proteins.[1,4] As there is a myriad of signalling molecules, regulated protein–protein interactions are one of the critical mechanisms for regulating specificity in signal transduction. Over the past decades a large number of proteins have been reported to be recruited to the TCR–CD3 complex. Here, we review recent data on five (direct or indirect) interaction partners of the TCR–CD3 complex, including the lymphocyte-specific protein tyrosine kinase (Lck), CD3ζ-associated protein kinase of 70 000 MW (ZAP-70), non-catalytic region of tyrosine kinase (Nck), Wiskott–Aldrich syndrome protein (WASP), and the inhibitor of Notch-1 signalling Numb (Table 1). Other proteins associated with the TCR–CD3 complex have been discussed elsewhere and they are not covered in this review.[8–13] The effects of some mutations of these proteins on TCR signalling is shown in Table 2.

T cells develop in the thymus where self-reactive T cells are deleted by a process called negative selection, which is based on a strong signal elicited by high-affinity binding to the self-peptide MHC.[14,15] A lack of, or mutation in, the critical proteins involved in TCR–CD3 signalling, such as ZAP-70 and WASP, causes a reduction of the TCR–CD3 signalling strength that allows autoreactive T cells to escape from negative selection and reach peripheral tissues.[16–20] These autoreactive T cells can be activated in response to self-peptide, which consequently leads to tissue injury known as autoimmune disease.[15,21] Hence, chemical agents blocking specifically the T-cell activation process are promising therapeutic interventions for the treatment of T-cell-driven diseases. Here, we cover the information on some inhibitors that target the signalling proteins at the TCR–CD3 as they may have a potential to be used for the treatment of autoimmune disorders and in organ transplantations.

Lck

Members of the Src family of protein tyrosine kinases modulate signal transduction downstream of transmembrane receptors in most, if not all, cell types. In T cells, Lck is a member of the Src family of 56 000 MW. TCR–CD3 engagement with an antigenic peptide MHC triggers the phosphorylation of the ITAM tyrosines by Lck.[22] Phosphorylated ITAMs then become a docking site for ZAP-70, which is also activated by Lck upon binding to the ITAMs.[23] Subsequently, ZAP-70, together with Lck, phosphorylates downstream signalling molecules, to activate TCR–CD3-controlled signalling cascades.

Lck contains an N-terminal membrane anchor region (SH4 domain), a unique domain, an Src-homology 3 (SH3) domain, an SH2 domain, a catalytic kinase domain and a short C-terminal tail (Fig. 1a). The SH4 domain is post-translationally modified by the addition of lipids, including myristoylation and palmitoylation, which allows the attachment of Lck to the plasma membrane. A serine 59 residue in a unique domain of Lck can be phosphorylated by the extracellular signal-regulated kinase (ERK)[24] and phosphorylation of this residue inhibits Lck activity.[25] In addition, Lck activity is tightly regulated by a conformational state mainly depending on the phosphorylation and dephosphorylation of two tyrosine residues (Y394 and Y505) on the catalytic kinase domain and the C-terminal tail, respectively.[26] Phosphorylation of Y505 by the C-terminal Src kinase mediates an intramolecular interaction with the SH2 domain, resulting in an inactive or closed conformation of Lck. When Y505 is dephosphorylated by the phosphatase CD45 or SHP-1, the SH2 domain detaches from Y505, so promoting an opened

Table 1. Selected proteins interacting with the T-cell receptor (TCR) –CD3 complex

Proteins associated with TCR–CD3	Binding domain of the associated protein	Binding motif of the TCR–CD3	Effect on TCR signalling	References
Lck	SH2	Phospho-ITAM	Enhancement	38
ZAP-70	SH2	Phospho-ITAM	Enhancement	86
Nck	SH3.1 and SH2	Proline-rich sequence (PRS) and Phospho-ITAM within CD3ε	Enhancement	40, 65
WASP	SH3 domain bind to Nck	Indirect via Nck	Unknown	79
Numb	Phosphotyrosine binding (PTB) domain	NPDY motif within CD3ε	Decrease	84

Table 2. Mutations of T-cell receptor (TCR) -CD3 binding proteins with their effects on TCR signalling

TCR–CD3 binding proteins	Mutations	Effects on TCR signalling	References
Lck	R154K (SH2 mutant)	Inhibits Lck association with ZAP-70 and CD3ζ	38
	Y192F	Inhibits Lck association with TSAd, Itk, Pyk2 and SHP-1 and enhances tyrosine-phosphorylated proteins	87
	Y394F	Closed conformation with decreased kinase activity	35,88
	Y505F	Open conformation with increased enzymatic activity	35,88
	Y505F, K273R	Open conformation but lacking kinase activity	35
ZAP70	Y315F	Inhibition of Vav–ZAP-70 interaction and reduction of tyrosine phosphorylation	89
	Y319F	Impairment of Ca^{2+} mobilization, Ras activation and activation of phospholipase Cγ1	54
	W131A	Increases kinase activity of ZAP-70	90
	Y315, 319A	Open conformation with increased kinase activity of ZAP-70	51
	Y315, 319F	Closed conformation with ZAP-70 kinase inactive	51
	D461N	Inactivates the kinase domain known as 'kinase dead'	50
	Y493F	Inactivates ZAP-70 catalytic activity	91
Nck	Nck1(W38K) (SH3.1 mutant)	Impairs the binding of Nck1 to CD3ε and decreases ERK activation	65
	Nck1(W143K) (SH3.2 mutant)	Impairs the binding of Nck1 to Cbl	92
	Nck1(W229K) (SH3.3 mutant)	Impairs the expression of CD69 expression and ERK phosphorylation	63
	Nck1(R308K) (SH2 mutant)	Impairs the binding of Nck1 to CD3ε Abrogates the binding of Nck1 to ADAP	65,93
WASP	WASPΔC (deletion of amino acids 444–502 at C terminus)	Inhibits actin polymerization but enhances the activation of NFAT transcription factor and ERK phosphorylation in human T cells	94
	L46P (WH1 mutant)	Impairs the chemotactic migration of human T cells and actin cytoskeleton reorganization	95
	A47D (WH1 mutant)	Impairs the chemotactic migration of human T cells and actin cytoskeleton reorganization	95
Numb	ΔNumb (condition deletion of *Numb*)	Normal CD3ζ phosphorylation in murine T cells	96

conformation. The opened conformation allows phosphorylation of Y394 by Lck trans-autophosphorylation.[26,27] However, doubly phosphorylated tyrosines Y394 and Y505 might also exist and confer a dominant effect of kinase activity over the inhibitory Y505.[28]

Different pools of Lck have been identified, including Lck in the cytoplasm, Lck anchored to the plasma membrane and Lck associated with the co-receptors CD4 and CD8.[29] Approximately 40% of Lck is already active (phosphorylated at Y394) in resting T cells and upon TCR engagement the amount of active Lck increases, as seen by Forster resonance energy transfer.[30–32] In addition, the distribution of Lck to its correct destination may regulate the function of Lck in phosphorylating its substrates.[26] Several lines of evidence have also suggested that initial phosphorylation of the CD3's ITAMs is mediated by free Lck, whereas the co-receptor-associated Lck acts as an adaptor molecule to bring the CD4 or CD8 molecule to the phosphorylated TCR–CD3 complex in a later step.[33,34] Furthermore, it has been suggested that the conformation of Lck determines its distribution. Lck in the opened conformation might allow clusters of Lck, whereas the closed conformation inhibits the clustering. TCR triggering induces the clustering of Lck with the phosphorylated TCR–CD3, suggesting that conformation-driven Lck clustering may determine its localization to perform its activity.[35] These findings suggest that Lck recruitment to the TCR–CD3 complex induces the phosphorylation of the ITAMs.

Lck can be co-immunoprecipitated with the TCR–CD3 complex upon TCR–CD3 ligation, suggesting that these two proteins can interact directly or indirectly with each other.[36] An indirect interaction might be mediated by RhoH, a haematopoietic-specific Rho GTPase.[37] The direct interaction might be mediated by the SH2 domain of Lck and phosphorylated ITAMs.[38,39] In addition, the SH2 domain of Lck can also interact with lipid within the plasma membrane upon TCR activation. This binding might be crucial for a lateral diffusion of Lck to interact with the triggered TCR–CD3 complex.[39] These data indicate that localization of Lck to TCR–CD3s that are phosphorylated on few tyrosines facilitates the phosphorylation of the other ITAM tyrosines within CD3.

Our own data have suggested that the resting TCR–CD3 is in a closed conformation, in which the ITAM tyrosines are not exposed, but hidden within the quartenary structure of the TCR–CD3 complex.[40,41] Upon peptide–MHC binding to TCR-αβ an open CD3

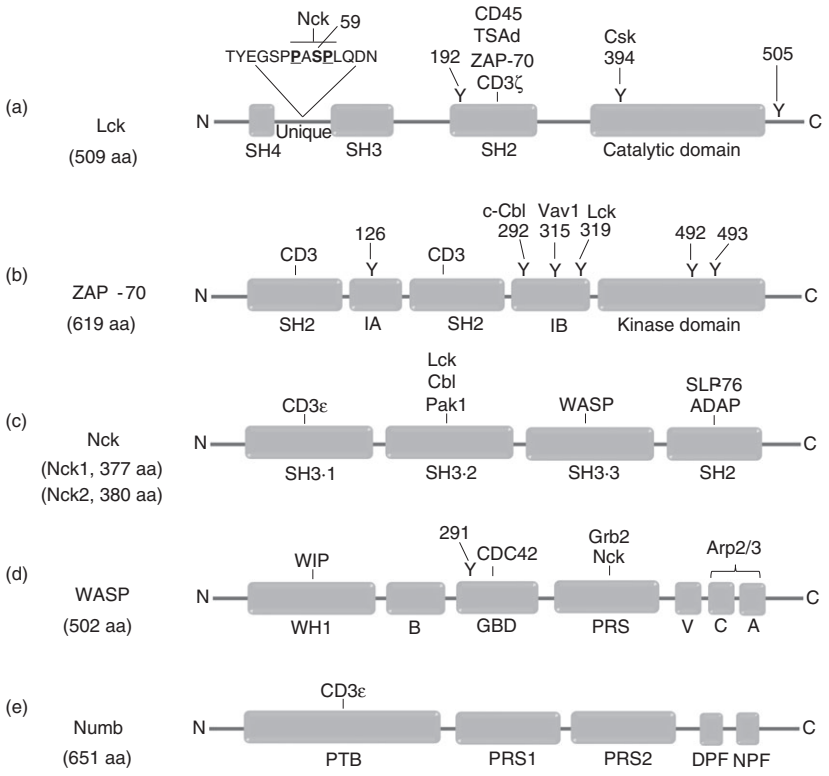

Figure 1. Modular composition of proteins associated with the T-cell receptor (TCR) –CD3 complex. (a) Lck consists of an Src homology 4 (SH4) domain, a unique domain, an SH3 and SH2 domain, the catalytic domain and a C-terminal region. The serine (S) and tyrosines (Y) depicted can be phosphorylated upon TCR–CD3 ligation. (b) ZAP-70 contains an N-terminal SH2 domain, an interdomain A (IA), a C-terminal SH2 domain, an interdomain B (IB) and the kinase domain. The tyrosine residues indicated can be phosphorylated upon TCR–CD3 triggering. (c) The Nck family has two members, Nck1 and Nck2, both being composed of three SH3 domains and a C-terminal SH2 domain. (d) WASP consists of a WH1 (WASP homology 1) and basic domain, followed by a GTPase-binding domain (GBD), a proline-rich sequence (PRS) and ver-prolin homology domain–cofilin homology domain-acidic region domains (VCA). (e) Numb contains a phospho-tyrosine binding (PTB) domain, two PRSs and DPF (Asp-Pro-Phe) and NPF (Asn-Pro-Phe) tri-peptide motifs at the C-terminus.

conformation is stabilized, that allows access of Lck to the ITAM tyrosine.[42] This might be one explanation of how peptide–MHC binding to the TCR–CD3 complex causes CD3 phosphorylation by Lck.

As Lck expression is found only in T cells and natural killer cells, selective inhibitors that target Lck would potentially provide a safe treatment of diseases mediated by over-activation of T cells such as rheumatoid arthritis, inflammatory bowel disease, psoriasis and organ graft rejection.[43] A large number of compounds have been reported that selectively inhibit Lck activity by binding to the ATP pocket of Lck's kinase domain.[44] Some of those inhibitors prevent the allograft rejection in mouse models,[45,46] and one inhibits the hind paw swelling in an adjuvant-induced rat arthritis model.[47]

ZAP-70

ZAP-70 is a cytoplasmic tyrosine kinase expressed predominantly in T and natural killer cells. The importance of ZAP-70 in humans has been demonstrated as a lack of ZAP-70 causes a profound combined immunodeficiency, which is characterized by an absence of CD8 T cells and a defective function of CD4 T cells.[17,18] Combined mutations of R192W and R360P in ZAP-70 cause an autoimmune syndrome. The former mutation results in decreased binding to phospho-CD3, whereas the latter mutation reduces an autoinhibitory mechanism.[48] These mutations that alter TCR signalling thresholds cause autoimmune diseases as phenotypically demonstrated by uncontrollable bullous pemphigoid, colitis and proteinuria.[48]

ZAP-70 is structurally composed of two SH2 domains separated by a so-called interdomain A. Following the tandem SH2 domains is the interdomain B and the kinase domain[49] (Fig. 1b). There are several tyrosine residues on the interdomain B and kinase domain that can be phosphorylated after TCR stimulation. These tyrosines have various functions including regulation of the catalytic activity of ZAP-70 and interaction with other signalling molecules. Tyrosine 292 (Y292), Y315 and Y319 are located within the interdomain B, whereas Y492 and Y493 are located in the kinase domain. In resting T cells, ZAP-

70 is in an autoinhibited conformation mediated by the intramolecular interaction of Y315 and Y319 with the kinase domain.[50] Upon TCR engagement, the tandem SH2 domains of ZAP-70 are recruited to doubly phosphorylated ITAMs of the CD3 subunits. Binding to the CD3 subunits changes ZAP-70 conformation to an opened conformation with the release of Y315 and Y319 from the kinase domain. This facilitates the phosphorylation of Y315 and Y319 by either Lck[51,52] or by trans-autophosphorylation.[53] Likewise, the conformational change also gives rise to a more flexible kinase domain, resulting in phosphorylation of Y493, which is located within the activation loop of the kinase domain, by either Lck or by trans-autophosphorylation.[51] Phosphorylation of Y493 allows ZAP-70 to be catalytically active. Lck can bind with its SH2 domain to phospho-Y319 of ZAP-70 and is required to mediate the phosphorylation of various tyrosine residues on ZAP-70.[54] Mutation of ZAP-70's Y319[53] or Lck's SH2 domain[54] abrogates the Lck–ZAP-70 interaction and consequently impairs downstream signalling. Taken together, the activation of ZAP-70 relies on two steps: first binding of the tandem SH2 domains of ZAP-70 to doubly phosphorylated tyrosines within the ITAMs of CD3, causing a conformational change, and second the Lck- and ZAP-70-mediated phosphorylation of Y315, Y319 and Y493 resulting in full ZAP-70 activation.[49–51]

By comparing the different ITAMs among the CD3 subunits (CD3ζ, CD3δ, CD3ε and CD3γ), it is likely that ZAP-70 preferentially binds to fully phosphorylated CD3ζ.[9] Recently, a 'catch-and-release' model for ZAP-70 activation has been proposed by Katz *et al.*[55] After recruitment of ZAP-70 to the phosphorylated TCR–CD3 complexes and ZAP-70 phosphorylation by Lck, activated ZAP-70 is released from the TCR–CD3 complexes into the plane of the plasma membrane. The association of ZAP-70 with the membrane might be mediated by the binding of the SH2 domains to lipids or of phosphotyrosines to other membrane-associated proteins. Consequently, empty phospho-TCR–CD3 complexes allow the recruitment of additional ZAP-70 molecules to the TCR–CD3 for activation of additional ZAP-70. The released ZAP-70 translocates within the membrane into adjacent protein islands to mediate phosphorylation of its substrates including the linker for the activation of T cells (LAT) and the SH2-domain-containing leucocyte protein of 76 000 MW (SLP-76).[55] Phosphorylated LAT and SLP-76 adaptor proteins have various interacting partners such as the phospholipase C-γ1, which is recruited to these two proteins to form the LAT/SLP-76 signalosome.[56] Forming of this signalosome results in T-cell activation, proliferation and differentiation.

As ZAP-70 is required to initiate T-cell activation, inhibition of ZAP-70 from interacting with the TCR–CD3 by small molecules may be used to treat patients with autoimmune diseases and organ transplants. High-throughput screening of a library of 132 842 compounds has been conducted to find inhibitors that would disrupt the interaction of ZAP-70 with CD3ζ.[57] A series of pyrimidine derivatives that can inhibit ZAP-70 activity have been identified and patented by researchers and Novartis companies.[58]

In recent years, chimeric antigen receptor (CAR)-expressing T cells have been used for tumour immunotherapy. CARs consist of an extracellular anti-tumour antigen single Fv fragment, a transmembrane region and the cytoplasmic tail of CD3ζ. CAR signalling relies on tumour antigen-binding-induced CD3ζ tail phosphorylation. An *in silico* model has suggested that the sensitivity of TCR signalling is modulated by the differential affinities of ZAP-70 to the ITAMs of CD3ζ, and sequential phosphorylation of these ITAMs leading to a 'switch-like' response of TCR signalling.[59] Cytokine production by T cells could occur without phosphorylation of the CD3ζ, CD3δ, CD3γ chains when there are intact CD3ε chains.[60] It has been suggested that no matter which ITAMs are phosphorylated, the number of ITAMs to be phosphorylated would determine the outcome of the T-cell response.[61] Hence, to obtain effective CAR-T cells with a strong anti-tumoral cytotoxic function but without producing too much cytokine, preventing the so-called cytokine storm, one may optimize CD3ζ signalling by titrating the number of ITAMs to be phosphorylated and by using other CD3 chains than CD3ζ.

Nck

Nck is a 47 000 MW cytosolic adapter protein that is composed of three SH3 domains (SH3.1, SH3.2 and SH3.3) and one SH2 domain (Fig. 1c). In humans, two Nck isoforms exist; Nck1/Nckα and Nck2/Nckβ, which share 68% amino acid sequence similarity.[62] Although redundant roles of Nck1 and Nck2 have been reported, our previous work has shown that Nck1 and Nck2 molecules are functionally non-redundant in T-cell activation.[63] In response to TCR triggering, Nck is recruited to SLP-76 to mediate actin rearrangement, which is essential for immunological synapse formation, T-cell activation and cell movement.[64] Nck is doing so by binding to WASP.

In addition, inducible direct association of Nck to the TCR–CD3 complex occurs when the latter is triggered. For this association, Nck simultaneously uses its SH3.1 and SH2 domains.[65] The SH3.1 domain directly interacts with the PxxPxxDY sequence located within the proline-rich sequence (PRS) of the CD3ε.[40] For this association to occur the TCR needs to be in its *Active* CD3 conformation and the tyrosine needs to be in the non-phosphorylated state.[40,66] The SH2 domain interacts with the second tyrosine of the CD3ε ITAM, when this tyrosine is phosphorylated.[65] The functions of Nck–CD3 interaction is not well understood. A knock-in mouse

strain was generated in which the CD3ε PRS was replaced with another sequence, abolishing the binding to the SH3.1 domain of Nck, but most likely also to Numb (see below).[67] The fact that Nck is a positive and Numb a negative regulator of signalling, might explain why the phenotype of the mutant T cells was mild. To only block the Nck–CD3 interaction, another knock-in mouse line with point mutations of the two central prolines of the PxxP motif of CD3ε PRS to alanine has been generated.[68] Indeed, T cells from these knock-in mice do not recruit Nck to the TCR upon stimulation. In addition, this mutation is accompanied with impaired CD3ζ phosphorylation and decreased ZAP-70 recruitment to the TCR–CD3 complex, as well as impaired ZAP-70 phosphorylation.[68] Moreover, the SH3.2 domain of Nck can bind to a proline motif in the unique domain of Lck.[69] Recently, another adaptor protein called the T-cell specific adaptor protein (TSAd) was identified that interacts with the Src family of proteins including Lck and promotes actin polymerization via interaction with Nck.[70] Nck and Lck contain multiple binding sites on TSAd. The Nck SH2 interacts with phospho-TSAd whereas the Nck SH3.1 and SH3.3 interact with TSAd PRS. The SH2 Lck binds to phospho-TSAd and the Lck SH3 binds to the TSAd PRS. Taken together, Nck recruitment to the TCR–CD3 complex may also bring Lck to TCR.

Interestingly, the importance of the Nck–CD3 interaction might depend on the antigen quality, as this interaction was critical for stimulation of T cells with weak (low-affinity) antigens, but not with strong (high-affinity) antigens.[71] Foreign antigens are often of high affinity and self antigen of low affinity.[72] Hence, the requirement of Nck recruitment for T-cell activation only by low (and not by high) affinity antigens has raised the possibility for inhibition of the Nck–CD3 interaction as a target for treatment of autoimmune diseases caused by self-reactive T cells. Borroto *et al.*[73] have chemically generated a low-molecular-weight inhibitor targeting a non-canonical pocket within the Nck SH3.1 domain. As expected, this inhibitor prevented the binding of Nck to the TCR–CD3 complex. T-cell activation in response to low-affinity antigens was strongly inhibited by this inhibitor, as seen in mouse models for psoriasis, asthma and multiple sclerosis. Interestingly, the T-cell response to a mouse pathogen acting as a strong high-affinity peptide was normal after treatment with this inhibitor. Altogether, these results indicate that this synthetic inhibitor could be a candidate to be evaluated in clinical trials to treat various T-cell-mediated autoimmune diseases.[73]

WASP

WASP belongs to the WASP family of proteins consisting of WASP, N-WASP and WAVE/SCAR molecules.[74] Mutation of WASP or lack of WASP expression causes the Wiskott–Aldrich syndrome (WAS), which is characterized by thrombocytopenia, eczema, increased susceptibility to infection and increased risk to develop autoimmune disease.[20,75] WASP contains a WASP homology 1 domain, a basic domain, a PRS, a GTPase-binding domain and a verprolin homology domain–cofilin homology domain-acidic region (VCA) domain (Fig. 1d). These domains are required for binding to different cytoskeleton-regulating proteins. For instance, the GTPase-binding domain binds CDC42,[76] whereas the PRS acts as a binding site for various SH3-containing proteins such as Nck.[77] The function of WASP at the SLP-76 signalosome in regulating actin skeleton dynamics is well described.[78]

As WASP is the binding partner of Nck,[77] we tested whether recruitment of Nck to the TCR–CD3 complex may also bring WASP to the TCR–CD3. We found that WASP is co-immunoprecipitated with the TCR–CD3 complex after T-cell activation.[79] However, whether this was mediated by Nck is not known. Although the function of WASP recruitment to the TCR–CD3 complex has not been investigated, these results suggest that there would be an alternative pathway of WASP (besides the SLP-76 signalosome) to regulate actin reorganization in the vicinity of the TCR–CD3 complex.

Numb

Numb is an adaptor protein that regulates receptor internalization. Numb is up-regulated in the active phase of multiple sclerosis[80] and type 1 diabetes.[81] Two homologues of Numb including Numb and Numb-like have been identified in mammals.[82] Numb is composed of a phosphotyrosine binding domain, several proline-rich regions at the centre of the molecule and two tri-peptide motifs (Fig 1e).[82] Numb is involved in the development of murine thymocytes by regulating pre-TCR signalling.[83] In addition, Numb may control TCR signalling in mature T cells. Constitutive expression of CD69 and interferon-γ, as well as constitutively phosphorylated ERK, are found in the $CD4^+$ T cells from dominant negative Numb transgenic mice. Upon stimulation, $CD4^+$ T cells from these mice exhibit higher ERK, ZAP-70 and Akt phosphorylation than those of the wild-type mice, indicating that Numb may be required for a negative control of TCR-mediated signal transduction.[84] It was suggested that Numb plays a role in TCR degradation by simultaneously binding to both Cbl and a site within CD3ε that overlaps with the Nck binding site, thus mediating TCR degradation.[84]

Numb can bind with its phosphotyrosine binding domain to the cytoplasmic tail of CD3ε within the PRS to the sequence NPDY.[84] Indeed, an endocytosis motif in CD3ε in this region has been identified,[85] suggesting that

Numb might be involved in TCR–CD3 endocytosis. Interestingly, Numb was suggested to constitutively associate with CD3ε. So far, not much is known about the order of binding of the TCR–CD3 binding partners. Here, we propose that in resting T cells, CD3ε is occupied with Numb that impedes TCR signalling. Upon TCR ligation, a conformational change of the CD3ε may result in the release of Numb and exposure of the CD3ε PRS, which is the site that interacts with Nck. Recruitment of Nck to the TCR also brings Lck to the TCR to facilitate ITAM phosphorylation. Full ITAM phosphorylation then releases Nck so that ZAP-70 can bind. Once the TCR signal is transmitted, ZAP-70 is replaced by Numb to mediate TCR degradation and these cause a deviation of T-cell activation. However, further studies are required to elucidate the mechanism underlying Numb-regulated TCR signalling and the related TCR degradation pathways.

Conclusion

TCR–CD3 complex is the key molecule to initiate biochemical events in T-cell activation and differentiation that can lead to different outcomes, depending on the quantity and quality of the stimulus. Nevertheless, how stimulation of the TCR–CD3 complex can give rise to distinct outcomes still remains unclear. Based on the recent findings, we propose that distinct outcomes may be due to the different interaction partners to be recruited to the TCR–CD3 complex upon TCR–CD3 engagement (Fig. 2). These protein partners are involved in both enhance and decrease of TCR signalling and in different downstream signalling pathways. Lck can interact directly or indirectly with the TCR–CD3 complex and phosphorylate the ITAMs to initiate signal transduction. ZAP-70 directly interacts with the TCR–CD3 complex upon CD3 phosphorylation and activates downstream

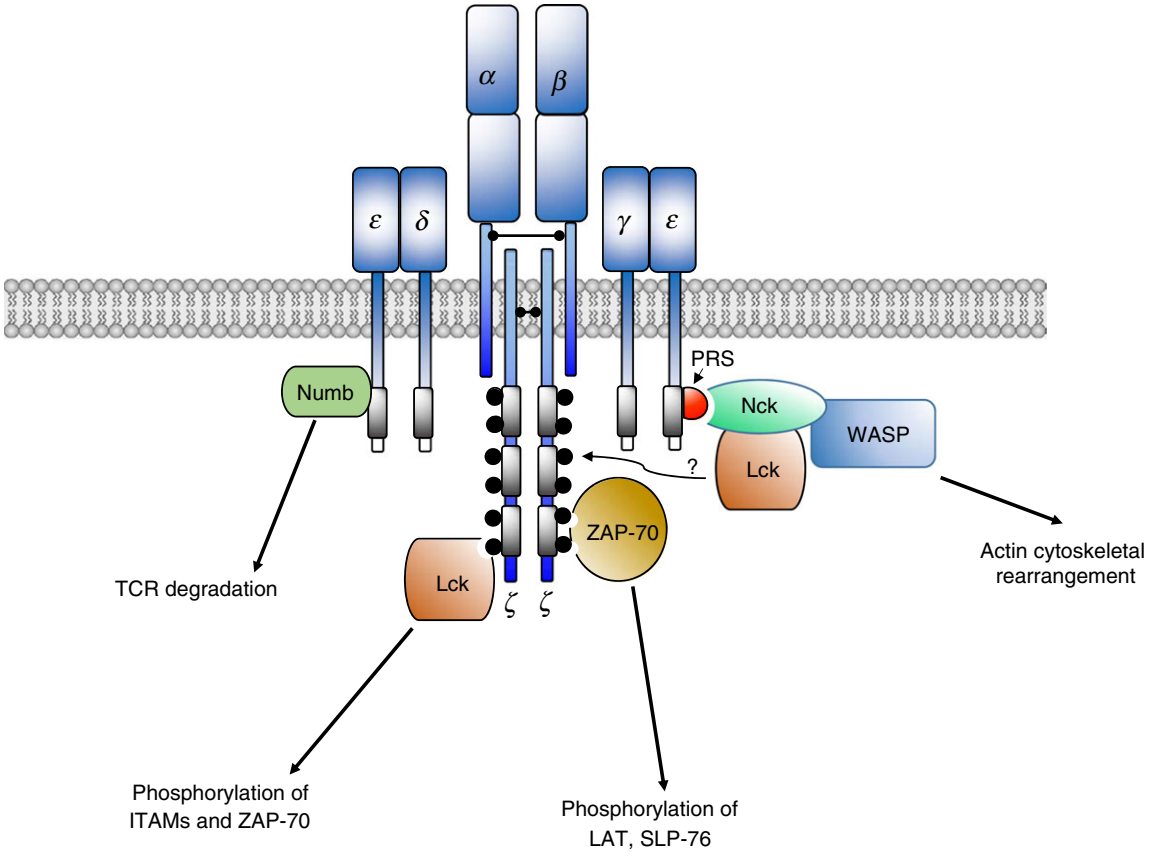

Figure 2. Selected signalling proteins at TCR–CD3 complex. TCR–CD3 ligation induces a conformational change of CD3ε, leading to the exposure of its proline-rich sequence (PRS). Nck is then recruited to the PRS within the cytoplasmic tail of the CD3ε. Subsequently, Lck is associated with Nck upon TCR activation. Hence, Nck recruitment to TCR may also bring Lck to TCR–CD3 complex to mediate phosphorylation of the ITAM motif. In addition, Lck can directly interact with phospho-ITAM. When the second tyrosine of the CD3ε ITAM is phosphorylated, Nck can bind with CD3ε using its SH3.1 and SH2 domains in a co-operative manner. In proximity to the TCR–CD3 complex, Lck phosphorylates tyrosines in each ITAM of the CD3 chains. ZAP-70 is then recruited to bind to the phospho-ITAMs, where ZAP-70 itself is phosphorylated by Lck. WASP can be associated with Nck upon TCR activation to regulate actin polymerization. Numb can be associated with the CD3ε to regulate in TCR degradation leading to a decrease in TCR signalling.

signalling cascades. Nck is recruited to CD3ε and might co-recruit Lck and WASP to the TCR–CD3 complex. TCR–CD3-recruited WASP might control actin reorganization at TCR–CD3. Numb is a new binding partner of the TCR–CD3 complex and participates in TCR degradation to lessen TCR signalling after T-cell stimulation. However, the exact molecular mechanisms underlying the dynamic distributions of these proteins into and out of the TCR–CD3 complex still need further clarification.

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Disclosures

The authors declare no conflict of interest.

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Nck Binds to the T Cell Antigen Receptor Using Its SH3.1 and SH2 Domains in a Cooperative Manner, Promoting TCR Functioning

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Ligand binding to the TCR causes a conformational change at the CD3 subunits to expose the CD3ε cytoplasmic proline-rich sequence (PRS). It was suggested that the PRS is important for TCR signaling and T cell activation. It has been shown that the purified, recombinant SH3.1 domain of the adaptor molecule noncatalytic region of tyrosine kinase (Nck) can bind to the exposed PRS of CD3ε, but the molecular mechanism of how full-length Nck binds to the TCR in cells has not been investigated so far. Using the in situ proximity ligation assay and copurifications, we show that the binding of Nck to the TCR requires partial phosphorylation of CD3ε, as it is based on two cooperating interactions. First, the SH3.1(Nck) domain has to bind to the non-phosphorylated and exposed PRS, that is, the first ITAM tyrosine has to be in the unphosphorylated state. Second, the SH2(Nck) domain has to bind to the second ITAM tyrosine in the phosphorylated state. Likewise, mutations of the SH3.1 and SH2 domains in Nck1 resulted in the loss of Nck1 binding to the TCR. Furthermore, expression of an SH3.1-mutated Nck impaired TCR signaling and T cell activation. Our data suggest that the exact pattern of CD3ε phosphorylation is critical for TCR functioning. ***The Journal of Immunology*****, 2016, 196: 448–458.**

Noncatalytic region of tyrosine kinase (Nck) is a ubiquitously expressed adapter protein that is composed of three SH3 domains (SH3.1, SH3.2, and SH3.3) and one SH2 domain, and thus it potentially interacts with proline-rich sequence (PRS)–bearing proteins and tyrosine phosphorylated proteins (1). In humans, two Nck isoforms exist (Nck1/Nckα and Nck2/Nckβ), which share 68% amino acid identity (2). Nck plays a pivotal role in actin reorganization, cell movement, and cell adhesion (3). Nck also acts as a linker for the recruitment and activation of other proteins in multiple intracellular signaling pathways (1, 2).

In αβ T cells, the TCR contains the TCRαβ heterodimer, which is responsible for Ag, that is, peptide–MHC, recognition. This heterodimer is noncovalently associated with two CD3 dimers (CD3δε, CD3γε) and a ζζ dimer, which are responsible for signal transduction (4, 5). The TCR bears 10 ITAMs (YXXl/I–$X_{6–8}$, YXXL/I) (6), a single ITAM in each CD3ε, CD3γ, and CD3δ, and three ITAMs in the ζ-chains (7). The phosphorylation of the tyrosine residues in the ITAMs can be promoted by the Src family kinase Lck and Fyn, resulting in the initiation of downstream signaling cascades (8). The doubly phosphorylated ITAMs recruit the tyrosine kinase ZAP70 by its tandem SH2 domains. Subsequently, ZAP70 phosphorylates adaptor proteins, such as SLP-76 and LAT (9, 10), thereby promoting signaling events, as for example Ca^{2+} mobilization and Erk and PI3K/Akt pathway activation. Phosphorylated SLP-76 can bind to the SH2 domain of Nck [SH2(Nck)], recruiting Nck to the "SLP-76 signalosome" (10–12).

It has been suggested that the TCR exists in two reversible conformations: the Ag-induced or "active conformation" and the "inactive conformation" adopted by nonengaged TCRs (13, 14). The peptide–MHC- or anti-TCR/CD3 Ab–induced conformational change at CD3 is required for TCR functioning (15, 16). For example, expressing a mutant CD3ε chain, in which a point mutation in the extracellular part inhibits the switch to the active conformation, results in a block of T cell activation (16) and T cell development (17).

In the active TCR conformation, the PRS in the cytoplasmic tail of CD3ε is exposed, and only in this conformation can the SH3.1

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The online version of this article contains supplemental material.

Abbreviations used in this article: Nck, noncatalytic region of tyrosine kinase; PLA, proximity ligation assay; PRS, proline-rich sequence; PV, pervanadate; sh, short hairpin; SV, streptavidin; WT, wild-type.

domain of Nck [SH3.1(Nck)] bind to the CD3ε PRS, as shown in a pull-down assay using a recombinant GST-SH3.1(Nck) fusion protein (13, 16, 18–22). Using biochemical approaches, it has been proposed that the recruitment of Nck to the TCR occurs earlier and independently of tyrosine phosphorylation (13). Another report has demonstrated that tyrosine phosphorylation is required for Nck recruitment to the plasma membrane (11); however, whether it was recruited to the TCR, to SLP-76, or to any other proteins remained unresolved. Whether tyrosine phosphorylation is needed for Nck recruitment is thus a matter of debate.

Recently, we have demonstrated that Nck1, rather than Nck2, plays a major role in TCR signaling in human cells (23). In the present work, we have focused on the functional role of Nck1 in cells conditionally lacking Nck2 to investigate the molecular mechanism governing the Nck–CD3ε interaction. Our data revealed that Nck needs both the SH3.1 and SH2 domains to bind to CD3ε. The requirement of tyrosine phosphorylation for CD3ε–Nck interaction was also studied using a pull-down assay with synthetic biotinylated CD3ε cytoplasmic tail peptides to mimic different CD3ε phosphorylation patterns. We show that Nck recruitment is dependent on a specific pattern of CD3ε tyrosine phosphorylation. Taken together, we suggest that the cointeraction of the SH3.1 and SH2 domains of Nck with partially phosphorylated CD3ε is required for efficient Nck binding and TCR phosphorylation.

Materials and Methods

Reagents and Abs

In this study, the following Abs were used: rabbit anti-Nck1, phospho-AKT (S473), phospho-ERK (T202/T204), and phospho-ZAP70 (Y319) Abs were purchased from Cell Signaling Technology; the mouse anti-CD3 (OKT-3) Ab was from eBioscience; the anti-idiotypic TCR Ab (C305) was from Millipore; the anti-ZAP70 and goat anti-CD3ε M20 Abs were from Santa Cruz Biotechnology; the anti-GST Ab was from Bethyl Laboratories; the anti–streptavidin (SV)-HRPO Ab was from SouthernBiotec; and the anti–phospho-ζ Ab ($pTyr^{142}$) and the inhibitor of Src family kinases PP2 were from Sigma-Aldrich (St. Louis, MO). The anti-ζ antiserum 448 and the anti–phospho-CD3ε antiserum (anti–phospho-εY1) have been described (24, 25). The below-mentioned peptides were obtained from Eurogentec: pepεPP, YWSKNRKAKAKPVTRGTGAGSRPRGQNKERPPPVPNPD**Y**EPIRKGQRDL**Y**GLNQRAV; pepγPP, AGQDGVRQSRASDKQTLLQNEQL**Y**QPLKDREYDQ**Y**SHLQGNQLRKK; and pepδPP, GHETGRPSGAAEVQALLKNEQL**Y**QPLRDREDTQ**Y**SRLGGNWPRNKKS (the Y in bold is phosphorylated).

Generation of double Nck1/2 knock-down cells and Nck1 mutants

Jurkat cells were cultured and expression of Nck1 and Nck2 was silenced simultaneously as previously described (23, 26). Briefly, cells were cotransfected with 0.5 μg pLVX–short hairpin (sh)Nck1 and pLVX-shNck2. Stable double Nck1/2 knock-down cells were selected by culturing the transfected cells in medium supplemented with 1.0 μg/ml puromycin and 1 mg/ml G418. The single clone was obtained from these lines by limiting dilution. For construction of double Nck1/2 knock-down cell-expressing Nck1 mutants, human Flag-tagged Nck-1 cDNA contained in the pEBB plasmid, provided by Prof. Bruce J. Mayer (Connecticut University Health Center, Farmington, CT), was used as a mutagenesis template. The wild-type Nck1 (WT-Nck1) and its mutant constructs, SH3.1*-Nck1 (W38K) and SH2*-Nck1 (R308K), were generated from the Nck1-pEBB plasmid using sited-directed mutagenesis with the QuickChange mutagenesis kit (Stratagene) using the following primers: 5′-GTGACCATGTGGGTTCCCTCTCCGAGAAATTAGCAGC-3′ and 5′-GCTGCTAATTTCTCGGAGAGGGAACCCACATGGTCAC-3′ for WT-Nck1, 5′-GCTTCTGGATGATTCTAAGTCCAAGTGGCGAGTTCGAAATTCC-3′ and 5′-GGAATTTCGAACTCGCCACTTGGACTTAGAATCATCCAGAAGC-3′ for SH3.1*-Nck1, and 5′-GGGGATTTCCTCATTAAGGATAGTGAATCTTCGCC-3′ and 5′-GGCGAAGATTCACTATCCTTAATGAGGAAATCCCC-3′ for SH2*-Nck1. The introduction of the expected mutation was confirmed by DNA sequencing. The sites of the mutated residues have been previously indicated as being important for ligand binding (27, 28), but without effect on the binding of the other unmutated domains (29). Double Nck1/2 knockdown cells were cotransfected with either plasmid-expressing WT-Nck1, SH3.1*-Nck1, or SH2*-Nck1 and pDsRed-Monomer-Hyg-N1 (0.1 μg DNA) (Clonetech), which provides resistance to hygromycin, with an electroporator using an Amaxa Nucleofector II and the manufacturer's protocol X-005. The transfectants were selected after 48 h in medium supplemented with 400 μg/ml hygromycin. Nck1 expression was assessed by immunoblotting. TCR expression was evaluated by flow cytometry, and for further studies, clones with similar expression levels were selected.

Cell stimulation, lysis, immunoprecipitation, and pull-down assay

Cells, including Jurkat, J.Cam1.6, as well as primary human $CD4^+$ T cells, were harvested and starved in RPMI 1640 medium without FBS for 1 h at 37°C. Jurkat and J.Cam1.6 cells were from the American Type Culture Collection (Rockville, MD). The primary $CD4^+$ T cells were isolated from buffy coats of the Blood Bank Centre of Naresuan University Hospital under the approval of Naresuan University Research Ethics Committee (Institutional Review Board no. 226/56). Subsequently, cells were either stimulated with the anti-TCR Ab C305 (1:50) for the indicated time points or left untreated. The Ab dilution (1:50) used in this study corresponds to ~0.12 μg/ml C305. For pervanadate (PV) treatment, cells were stimulated 1 mM PV for the indicated time points. In some experiments, cells were pretreated with 20 μM Src kinase inhibitor PP2 for 30 min at 37°C before stimulation. Cells were then lysed in 100 μl lysis buffer (20 mM Tris-HCl [pH 8], 137 mM NaCl, 2 mM EDTA, 10% glycerol, protease inhibitor mixture [Sigma-Aldrich]), 1 mM PMSF, 5 mM iodoacetamide, 0.5 mM sodium orthovanadate, 1 mM NaF, and 0.3% Brij96V) for 30 min on ice (30). TCRs from the total cellular lysates were either immunoprecipitated with 1 μg anti-CD3ε (OKT3) Ab-coupled protein–G Sepharose beads (Amersham Pharmacia Biotech), or pulled down with 4 μg of the indicated biotinylated CD3 peptide–coupled SV beads (Amersham Pharmacia Biotech). SDS-PAGE and immunoblotting were performed with the desired Abs, and visualization was done using a CCD camera (ImageQuant LAS 4000; GE Healthcare Life Sciences). Band intensity was assessed by the ImageJ software.

In situ proximity ligation assay

Jurkat or primary $CD4^+$ T cells were grown on diagnostic microscopic slides (Thermo Scientific). Cells were left unstimulated, stimulated with the anti-TCR Ab C305, stimulated with C305 in the presence of 20 μM PP2 (after PP2 pretreatment), or treated with 1 mM PV at 37°C for 5 min. Cells were then fixed with paraformaldehyde, permeabilized with 0.5% saponin, and blocked with blocking solution. Cells were then coincubated with the goat anti-CD3ε (Ab M20 [Santa Cruz Biotechnology]) and a rabbit anti-Nck1 Ab (Cell Signaling Technology). A proximity ligation assay (PLA) between the CD3ε and Nck1 molecules was performed according to the manufacturer's instructions with the Duolink kit (Olink Bioscience), resulting in red fluorescence signals. Cell nuclei were stained with DAPI. A confocal microscope (Nikon C2) was used for imaging and analysis. The number of the PLA signal dots was scored with the BlobFinder program (Uppsala University).

T cell activation assays

For the measurement of the kinetics of phosphorylation of AKT, MEK, and Erk, cells were stimulated with the anti-TCR Ab C305 (1:50) at 37°C for the indicated time points. SDS-PAGE and immunoblotting were performed with the Abs indicated, which were visualized using a CCD camera (ImageQuant LAS 4000; GE Healthcare Life Sciences). Quantification of the band intensities was assessed by the ImageJ software. To induce CD69 expression, 2×10^5 cells were starved overnight in a medium containing 1% serum at 37°C. Cells were incubated with the anti-TCR Ab C305 (1:50) at 37°C for 6 h and were then stained with an allophycocyanin-conjugated anti-CD69 Ab. The percentage of $CD69^+$ cells was analyzed on a Beckman Coulter CyAn ADP flow cytometer. FlowJo 6.1 software was used for data analysis.

Ca^{2+} influx measurement

Five million cells were resuspended in 1 ml RPMI 1640 medium supplemented with 1% FCS and labeled in the dark with 0.1% pluronic acid, 2.6 μM Fluo-3 AM, and 5.5 μM Fura Red AM (Life Technologies) for 30 min at 37°C. The stained cells were washed and kept on ice in the dark until the measurement. For calcium influx, cells were diluted 1:10 with prewarmed medium and maintained at 37°C during the event collection on a Beckman

Coulter CyAn ADP flow cytometer. Baseline fluorescence was monitored for 30 s, and then either medium or C305 (1:50) was added and the stimulation was recorded for 10 min. Data were analyzed with the FlowJo 6.1 software.

Statistical analysis

Data are represented as means ± SEM. All differences between experimental groups were analyzed with the Student *t* test. Significant differences were considered when the *p* values were <0.05.

Results

Active Lck is required for the interaction between Nck and the TCR upon TCR engagement

The binding of the TCR to beads coupled to the SH3.1(Nck) domain has been studied extensively (13, 16, 18, 19). However, the molecular requirements for the interaction between the TCR and endogenous Nck have not yet been fully explored. To determine whether the binding of Nck to the TCR is dependent on signal transduction, Jurkat cells were either pretreated with the Src tyrosine kinase inhibitor PP2 to prevent TCR phosphorylation by Lck and Fyn, or left untreated. Subsequently, cells were stimulated with the anti-idiotypic, anti-TCR Ab C305 or left unstimulated. In situ PLA is a technique that allows visualization (by a red fluorescent dot) of close proximity between endogenous proteins in fixed cells (31). Using PLA, we investigated the proximity of Nck with the cytoplasmic tail of CD3ε. Significantly more Nck–CD3ε PLA red dots were detected in stimulated compared with unstimulated cells (Fig. 1A), suggesting that endogenous Nck was recruited to the TCR upon TCR stimulation. In sharp contrast, PP2 treatment prevented the C305-induced proximity, indicating that Src kinase activity is necessary for the interaction of endogenous Nck to the triggered TCR. As a control, there was no PLA signal detected in the absence of either the anti-CD3ε or the anti-Nck1 primary Ab (Fig. 1A).

To test whether the induced Nck–TCR proximity was caused by Nck binding to the TCR, Jurkat cells were stimulated under the same conditions as in Fig. 1A and subjected to immunoprecipitation with anti-CD3 Abs. Consistent with the data from the PLA, Nck binding to the TCR was increased upon TCR triggering (Fig. 1B). Importantly, lower amounts of Nck were coimmunoprecipitated with the TCR from C305-stimulated and PP2-pretreated cells compared with C305-stimulated cells alone (Fig. 1B). As expected, PP2 was able to prevent tyrosine phosphorylation at CD3ε after TCR Ab–mediated engagement. When the coimmunoprecipitation experiment was performed using Lck-deficient Jurkat cells (J.Cam1.6), there was no increase of the TCR–Nck interaction upon C305 stimulation (Fig. 1C). Collectively, these data indicate that Lck activity is required for an efficient recruitment of Nck to the TCR.

Next, we performed a time course experiment of Nck coimmunopurification with the TCR using C305 as a TCR stimulus (Fig. 1D). At early time points (1 and 5 min) the increase in the association of Nck to the TCR correlated with the increase in CD3ε phosphorylation. However, at 10 min of stimulation, CD3ε phosphorylation still increased, whereas Nck recruitment decreased. This indicated that the recruitment of Nck might depend on another interaction besides the one using tyrosine phosphorylation of CD3ε. As before, stimulation of cells, in which tyrosine phosphorylation was inhibited by PP2, resulted in significantly weakened, but not absent, Nck recruitment. This might have been due to low levels of Lck activity or to binding of the SH3.1 domain of Nck to the PRS of CD3ε. Moreover, the requirement of Src kinase activity for Nck binding to the TCR was also observed in primary $CD4^+$ T cells (Fig. 1E, 1F), because the Nck–TCR interaction was sharply declined in the presence of PP2.

Taken together, these data suggest that active Src tyrosine family kinases, such as Lck, are critical mediators for Nck recruitment. Therefore, CD3 phosphorylation seems to be required for an efficient Nck–TCR interaction upon TCR stimulation.

PV treatment does not induce the Nck–TCR interaction

Having found that TCR phosphorylation is needed for an efficient Nck1–TCR interaction, we tested whether massive phosphorylations, as induced by the phosphatase inhibitor PV, are sufficient to induce Nck binding to the TCR. To this end, Jurkat cells were either stimulated with PV alone or with the anti-TCR Ab C305 and PV simultaneously, or with C305 alone, or left unstimulated. As before, the close proximity between Nck and CD3ε was investigated using PLA. First, PV alone was not able to induce close proximity between Nck and the TCR and, second, PV even inhibited the C305-induced Nck–TCR proximity (Fig. 2A).

Likewise, Nck was not copurified with the TCR upon PV treatment and PV inhibited the C305-induced copurification of Nck with the TCR (Fig. 2B). Phosphorylation of CD3ε was strongly induced by PV, suggesting that massive CD3ε phosphorylation might interfere with the Nck–TCR interaction. Furthermore, the inhibitory effect of PV on the Nck–TCR interaction was confirmed using primary human $CD4^+$ T cells (Fig. 2C, 2D), because the association of Nck to the TCR was dramatically decreased in the PV treatment.

Collectively, our data suggest that an intermediate level of tyrosine phosphorylation at CD3 is required for an efficient interaction between endogenous Nck and the triggered TCR.

Partial phosphorylation of CD3ε is required for efficient Nck binding

To test our hypothesis that only an intermediate pattern of tyrosine phosphorylation at CD3ε mediates Nck binding to the TCR, synthetic biotinylated peptides corresponding to the complete cytoplasmic tail of CD3ε were used (Fig. 3A): the nonphosphorylated CD3ε tail (ε), the CD3ε tail with the first cytoplasmic tyrosine phosphorylated (p39ε), with the second cytoplasmic tyrosine phosphorylated (p50ε), and the doubly phosphorylated CD3ε tail (ppε).

The different biotinylated CD3ε peptides were bound to SV-coupled beads, and their binding to the SH2(Nck), SH3.1(Nck), and full-length Nck as GST fusion proteins was tested in a pull-down assay (Fig. 3B). GST–SH2(Nck) was bound to all the phosphorylated CD3ε peptides (p39ε, p50ε, and ppε), but not to the nonphosphorylated peptide. In contrast, GST–SH3.1(Nck) was only bound to nonphosphorylated (ε) and the second tyrosine phosphorylated CD3ε peptide (p50ε) (Fig. 3B). These results are in line with our earlier findings that the SH3.1 domain binds to the PRS of CD3ε (13, 15), and that phosphorylation of the first tyrosine, which is located within the SH3.1 binding motif, inhibits SH3.1 binding (32–34). Interestingly, the GST–Nck full-length fusion protein was only bound to the p50ε peptide, suggesting that both an SH3.1–PRS (in the absence of Y39 phosphorylation) and an SH2–phosphotyrosine interaction were simultaneously needed for optimal Nck association (Fig. 3B).

Next, we repeated the CD3ε peptide pull-down assay using cellular lysates of Jurkat cells expressing endogenous Nck, instead of recombinantly expressed GST–Nck fusion proteins. Again, we saw that Nck bound strongly to the cytoplasmic sequence of CD3ε when the second tyrosine was phosphorylated alone (p50ε, Fig. 3C). Additionally, Nck bound weaker to the nonphosphorylated CD3ε (ε) peptide, indicating that the SH3.1(Nck) domain alone can mediate weak binding to CD3ε. Because the other CD3 subunits also contain phophorylatable tyrosines, we test

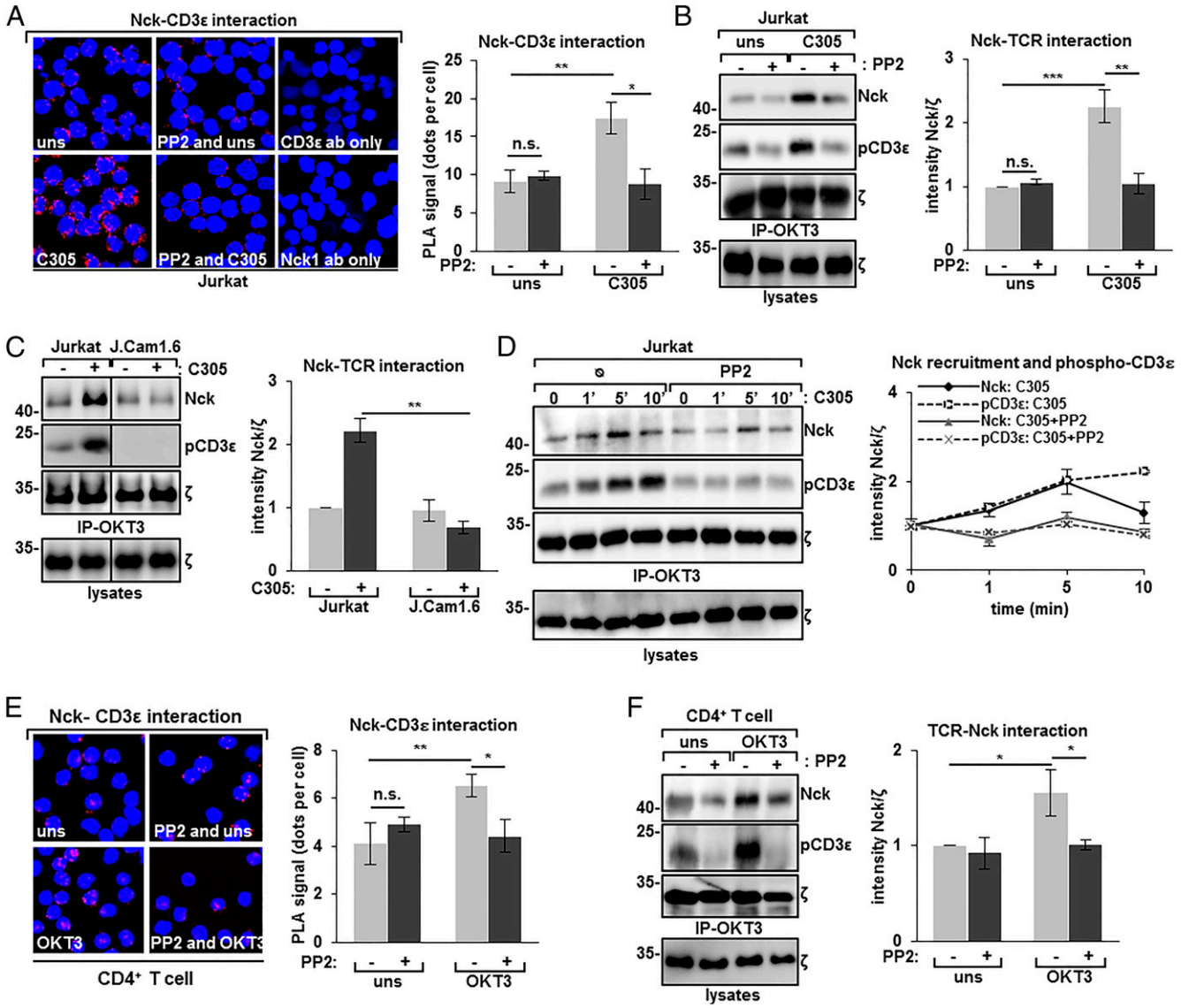

FIGURE 1. Lck activity is needed for Nck recruitment to the TCR. (**A**) Close proximity between the TCR and Nck was detected by in situ PLA. Jurkat cells were either pretreated with 20 μM PP2 or left untreated at 37˚C for 30 min. Subsequently, cells were stimulated with the anti-TCR Ab (C305, 1:50) with or without PP2 at 37˚C for 5 min or left unstimulated (uns). After fixation and permeabilization, PLA was performed using the primary Abs goat anti-CD3ε (M20) and rabbit anti-Nck1, as well as the corresponding secondary Abs. Negative controls, in which one of the primary Abs was omitted, were included (CD3ε Ab only, Nck1 Ab only). Nuclei were stained with DAPI. The corresponding quantification of the red PLA dots was scored from three independent experiments (data represent the mean ± SEM). (**B**) Jurkat cells were pretreated with PP2 and stimulated as in (A). Cell lysates were subjected to immunoprecipitation with the anti-CD3 Ab (OKT3). After SDS-PAGE, the Western blot was developed with anti-Nck1, anti–phospho-CD3ε (pCD3ε), and anti-ζ Abs. The corresponding lysates were developed with anti-ζ. Data are representative of five experiments. The intensity of the Nck1 and CD3ζ bands was quantified using ImageJ software and is presented as a ratio of Nck1 to CD3ζ normalized to the unstimulated cells (data represent the mean ± SEM). (**C**) Jurkat and J.Cam1.6 cells were either stimulated with C305 or left unstimulated, and immunoprecipitation with OKT3 was performed. SDS-PAGE, Western blotting, and quantification were done as in (B). Data are representative of five experiments. (**D**) Jurkat cells were either pretreated with PP2 or left untreated. Cells were then stimulated with C305 for the indicated times in the presence or absence of PP2. Immunoprecipitation, SDS-PAGE, and immunoblotting were done as in (B). Data are representative of at least two independent experiments. The intensity of the Nck1, pCD3ε, and ζ bands was quantified and is presented as a ratio of Nck1 or pCD3ε to ζ normalized to the unstimulated cells. (**E**) Primary human $CD4^+$ T cells were purified from buffy coats. Cells were either pretreated with 20 μM PP2 or left untreated at 37˚C for 30 min. Then cells were stimulated with 1 μg/ml OKT3 in the presence or absence of PP2 at 37˚C for 5 min or left unstimulated (uns). PLA was performed as in (A). The corresponding quantification of the red PLA dots was scored and analyzed as in (A). Data are representative of three independent experiments (mean ± SEM). (**F**) Primary human $CD4^+$ T cells were either left untreated or stimulated as in (E). Immunoprecipitation, SDS-PAGE, Western blotting, and analysis were done as in (B). Data are representative of three independent experiments. (A and E) Original magnification ×600. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

whether the SH2(Nck) domain might be sufficient to bind full-length Nck to CD3γ and CD3δ. To this end, we repeated the pull-down assay using the nonphosphorylated, singly phosphorylated, and doubly phosphorylated peptides corresponding to the complete cytoplasmic tails of CD3γ and CD3δ (Fig. 3D). We found that Nck could not bind to CD3γ and CD3δ, even when they carried full tyrosine phosphorylation. Next, we examined whether the simultaneous SH3.1–PRS and SH2–phosphotyrosine interactions have to take place in *cis*, that is, the PRS and phosphotyrosine have to be present within one CD3ε molecule, or can also take place in *trans*, that is, the PRS and the phosphotyrosine can be located on different CD3ε molecules. To this end, Jurkat lysates

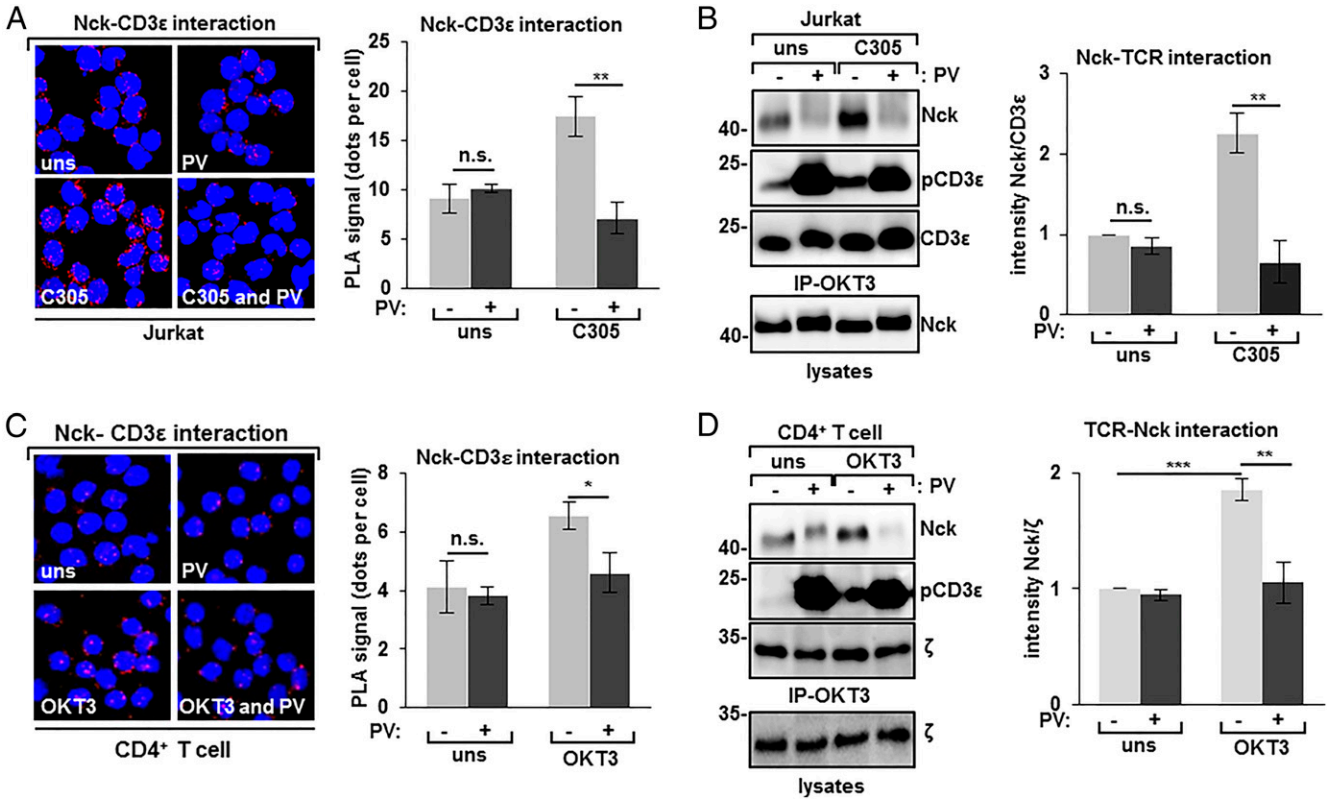

FIGURE 2. PV treatment does not induce Nck binding to the TCR. (**A**) Nck–TCR proximity was detected by in situ PLA. Jurkat cells were either stimulated with the anti-TCR Ab C305 with or without simultaneous treatment with PV, or with PV alone at 37°C for 5 min or left unstimulated. Experiment and analysis were done as in -. 1A. The corresponding quantification is shown as mean ± SEM of three independent experiments. (**B**) Jurkat cells were stimulated as in (A). Cellular lysates were subjected to immunoprecipitation with the anti-CD3 Ab OKT3. After SDS-PAGE the Western blot was developed with anti-Nck1, anti-pCD3ε, and anti-ζ Abs. The lysates were developed with anti-ζ. Data are representative of five experiments, and the statistical analysis was done as in Fig. 1B. (**C**) Primary human $CD4^+$ T cells were purified from buffy coats and either stimulated with 1 μg/ml OKT3 with or without simultaneous treatment with PV, or with PV alone at 37°C for 5 min or left unstimulated. PLA was performed as in Fig. 1A. The corresponding quantification of the red PLA dots was scored and analyzed. Data are representative of three independent experiments (mean ± SEM). (**D**) Primary human $CD4^+$ T cells were stimulated as in (C). Immunoprecipitation, SDS-PAGE, Western blotting, and analysis were done as in (B). Data are representative of three independent experiments. (A and C) Original magnification ×600. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

were used in a pull-down experiment with a mixture of the unphosphorylated CD3ε (ε, only allowing SH3.1 binding) and the double phosphorylated CD3ε peptides being bound to the SV-coupled beads. As a control, the unphosphorylated CD3ε peptide was mixed with the double phosphorylated CD3γ or CD3δ peptides (ppγ or ppδ), which cannot mediate SH2 binding (Fig. 3E). We kept the total amount of peptide constant in each sample. Endogenous Nck protein from the cell lysates bound better to the mixture of ε and ppε than to ε alone or ppε alone (Fig. 3E). Importantly, there was a synergistic effect, in that more Nck bound to ε and ppε than the sum of Nck binding to ε alone and ppε alone. This synergistic effect was not observed when ε was mixed with ppγ or ppδ (Fig. 3D). Collectively, these data suggest that Nck is able to interact with juxtaposed CD3ε, with one being non-phosphorylated and one being phosphorylated.

Nck requires the SH3.1 and SH2 domains to bind to the TCR

Because we found that partial CD3ε phosphorylation was required for an efficient Nck binding, we hypothesized that Nck might use both its SH3.1 and SH2 domains to bind to the TCR. Hence, we simultaneously knocked down the expression of Nck1 and Nck2 in Jurkat cells using Nck1- and Nck2-specific shRNAs (shNck1/2 cells). Nck1/2 protein expression in shNck1/2 cells was reduced by ∼80% compared with control-shRNA–transfected cells (see Supplemental Fig. 1). shNck1/2 cells were reconstituted with Flag-tagged Nck constructs encoding either WT Nck1 (WT-Nck1), or Nck1 in which the first tryptophan in the WW motif at position 38 within the SH3.1 domain was mutated to lysine (SH3.1*-Nck1), or Nck1 in which arginine at position 308 within the SH2 domain was mutated to lysine (SH2*-Nck1) (Fig. 4A). The W38K point mutation in the SH3.1 domain disrupts the interaction of Nck with proline-rich proteins, whereas the R308K point mutation in the SH2 domain mutant impairs binding to phosphorylated tyrosine residues (2). The SH3.1*-Nck1 protein was expressed to a similar level as the WT-Nck1 protein, and the expression level of both proteins was >5-fold increased compared with the remaining level of endogenous Nck1 after the knockdown (Fig. 4B, data not shown). In contrast, the SH2*-Nck1 protein was only weakly expressed, and had a similar expression level as endogenous Nck after the knock down (Fig. 4B). All cells expressed similar amounts of TCR on their cell surface as shown by flow cytometry (Fig. 4C).

The cell lysates of shNck1/2 and WT-Nck1– and SH3.1*-Nck1–expressing shNck1/2 cells were subjected to the pull-down assay using different synthetic CD3ε peptides as in Fig. 3 (Fig. 4D). There was a substantial binding of the WT-Nck protein to the p50ε peptide and a weaker binding to the nonphosphorylated ε peptide, corroborating our findings of Fig. 3. In sharp contrast, the SH3.1*-Nck1 mutant did not bind strongly to any peptide, indicating that the SH3.1(Nck) domain is crucial for Nck binding to the TCR. Again, endogenous residual Nck bound best to the p50ε (Fig. 4D, 4F). Next, we repeated this experiment using the SH2*-Nck1–

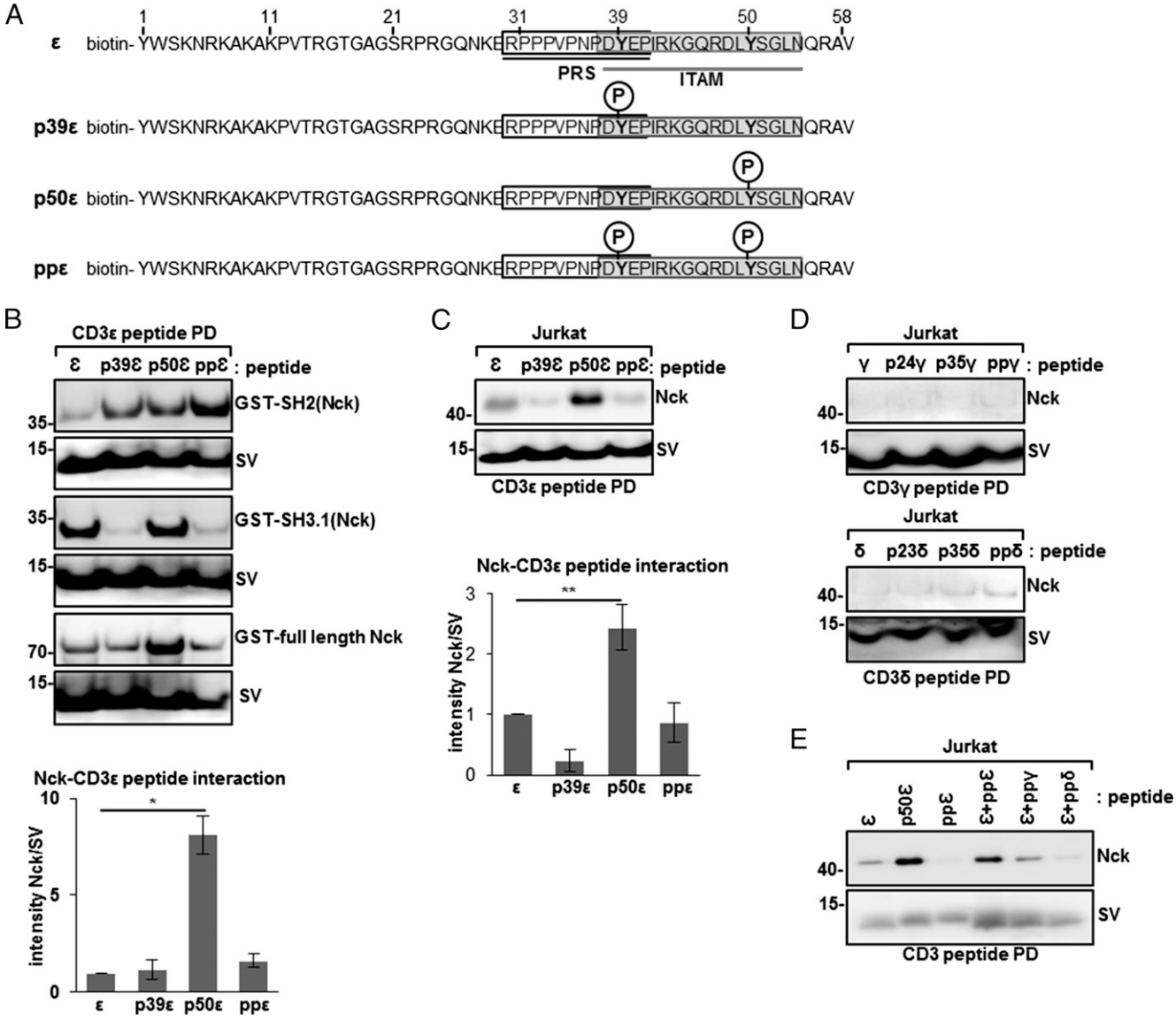

FIGURE 3. Only partially phopshorylated CD3ε can bind to Nck. (**A**) Schematic representation of the amino acid sequence of the different biotinylated CD3ε cytoplasmic tail synthetic peptides. Phosphorylations are indicated with a P. (**B**) The different synthetic CD3ε peptides shown in (A) were coupled to SV beads and incubated with the GST–SH2(Nck), GST–SH3.1(Nck), and GST–full-length Nck fusion proteins as indicated. Bead-bound proteins were separated by SDS-PAGE and the Western blot developed with an anti-Nck1 Ab as well as SV. Data are representative of three experiments. The intensity of the Nck and biotinylated peptide bands using full-length Nck was quantified using the ImageJ software and is presented as a ratio of Nck to peptide normalized to the value of the Nck/nonphosphorylated CD3ε peptide ratio (data represent the mean ± SEM). (**C**) Jurkat lysates were incubated with SV beads bound to different biotinylated CD3ε peptides. Bead-bound proteins were detected as in (B). The statistical analysis of Nck1 binding to the different CD3ε forms was done as in (B). (**D**) Jurkat lysates were incubated with SV beads bound to the indicated biotinylated CD3γ and CD3δ peptides. Beads-bound proteins were assessed as in (B). (**E**) Jurkat lysates were incubated with SV beads bound to the different biotinylated CD3ε peptides or to a mixture of unphosphorylated CD3ε and doubly phosphorylated CD3ε, CD3γ, or CDδ peptides as indicated. Bead-bound proteins and the lysates as controls were detected as in (B). $*p < 0.05$, $**p < 0.01$.

expressing cells (Fig. 4E, 4F). We found that, in contrast to the endogenous or WT-Nck, the SH2*-Nck1 protein could not bind strongly to the p50ε peptide, indicating that the SH2(Nck) domain is important for Nck binding to the TCR. Moreover, the binding to the unphosphorylated peptide was unchanged, indicating that the binding by the SH3.1 mutated was unaffected by the SH2 mutation. Altogether, these data indicate that Nck binding to the TCR crucially depends on the SH3.1 domain and it is stabilized or enhanced by the interaction of the SH2 domain with the phospho-Y50 of the CD3ε chain.

To further establish the loss of Nck recruitment to the TCR in cells that expressed the SH3.1*-Nck1 mutant, PLA was performed to determine the close proximity between SH3.1*-Nck and CD3ε molecules (Fig. 4G). There were a few PLA dots in shNck1/2 cells possibly due to residual endogenous Nck expression (Supplemental Fig. 1), which were used as a negative control. The number of PLA dots were significantly declined in SH3.1*-Nck1–expressing cells following TCR engagement as compared with stimulated WT-Nck1–expressing cells, corroborating our finding that the SH3.1(Nck) domain is critical for the Nck–TCR interaction. In stimulated SH3.1*-Nck1–expressing cells more PLA dots were detected than in stimulated shNck1/2 cells, suggesting that Nck was able to bind very weakly to the TCR through its SH2 domain (Fig. 4G). Consistent with the result from the PLA assay, the SH3.1*-Nck mutant was less coprecipitated with the TCR compared with the WT-Nck1 protein (Fig. 4H). Owing to the low

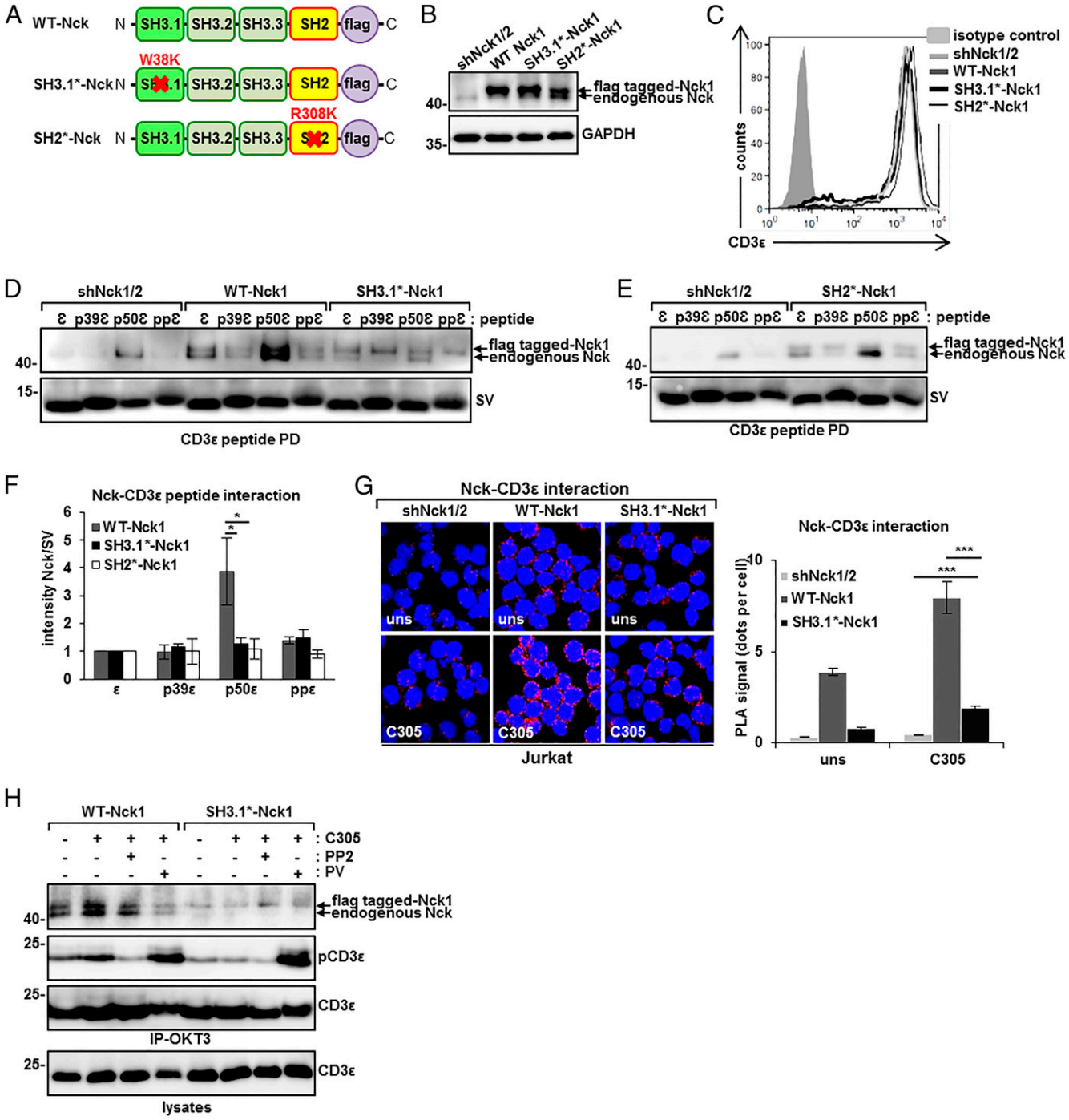

FIGURE 4. Nck needs the SH3.1 and SH2 domains to bind to the TCR. (**A**) Schematic illustration of Flag-tagged wild-type Nck (WT-Nck), SH3.1*-Nck (W38K) containing a non-functional SH3.1 domain, and SH2*-Nck (R308K) containing a nonfunctional SH2 domain. (**B**) Flag-tagged Nck1 expression from double Nck1/2 knock-down cells (shNck1/2) stably expressing WT-Nck1, SH3.1*-Nck1, or SH2*-Nck1. Lysates of the indicated cells were analyzed using immunoblotting with anti-Nck1 and anti-GAPDH Abs as a loading control. (**C**) Cells were stained with allophycocyanin-conjugated anti-CD3ε Ab UCHT1. Fluorescence intensities were measured by flow cytometry. (**D**) Lysates of shNck1/2 cells and WT-Nck1– and SH3.1*-Nck1–expressing cells were incubated with the indicated CD3ε peptides bound to SV-coupled beads. Bound proteins were detected as in Fig. 3. (**E**) Lysates of shNck1/2 and SH2*-Nck1–expressing cells were treated as in (E). (**F**) The statistics from three independent experiments as in (D) and (E) are presented as a ratio of Flag-tagged Nck to peptides normalized to the value of the Nck/nonphosphorylated CD3ε peptide ratio (data represent the mean ± SEM). (**G**) shNck1/2-, WT-Nck1–, and SH3.1*-Nck1–expressing cells were either left untreated or treated with anti-TCR (C305) Ab at 37˚C for 5 min. The close proximity between Nck and TCR in these cells was detected using in situ PLA as done in Fig. 1A. The corresponding quantification of PLA signal dots was collected from three independent experiments and analyzed as in Fig. 1A (data represent the mean ± SEM). Original magnification ×600. (**H**) The WT-Nck1– and SH3.1*-Nck1–expressing cells were treated with indicated treatments or left untreated. Cell lysates were subjected to immunoprecipitation with anti-CD3 Ab (OKT3) following immunoblotting with anti-Nck1, anti-pCD3ε, and anti-ζ Abs. $*p < 0.05$, $***p < 0.001$.

expression level of SH2*-Nck, we did not perform these experiments with the SH2 domain mutant Nck. Taken together, these findings indicated that Nck binding to the stimulated TCR is mediated by the SH3.1(Nck) and SH2(Nck) domains.

Impairment of the Nck–CD3ε interaction impairs T cell activation

It has been suggested that the PRS of CD3ε and its interaction with Nck are required for optimal signaling induced by the TCR (13, 22, 35). To substantiate these findings, we analyzed the involvement of Nck's interaction with the TCR for T cell activation, by using SH3.1*-Nck1–expressing shNck1/2 cells. The SH3.1*-Nck1 protein cannot bind to the TCR, but it retains other functions such as interactions using its SH3.2, SH3.3, and SH2 domains. WT-Nck1– and SH3.1*-Nck1–expressing shNck1/2 cells were stimulated with the anti-TCR Ab C305 in a time course experiment (Fig. 5A). The phosphorylation of CD3ε and recruitment of ZAP70 to the TCR were impaired in the shNck1/2 cells, but restored in the WT-Nck1 reconstituted cells, indicating that Nck is involved in these processes. A functional SH3.1(Nck) domain is important for these activities, because SH3.1*-Nck1 could not restore CD3ε phosphorylation or ZAP70 binding to the TCR. This suggests that Nck binding to the TCR is involved in CD3 phosphorylation.

Next we assessed whether the reduced signaling at the TCR also impaired downstream events. We found that the TCR-induced phosphorylations of Akt and Erk1/2 were strongly reduced in cells expressing SH3.1*-Nck1 compared with the cells expressing WT-Nck1 (Fig. 5B). A very weak phosphorylation of Erk in the SH3.1*-Nck1–expressing cells as well as in the control shNck1/2 cells was observed, indicating that this activity might be due to the low expression levels of endogenous Nck proteins (Supplemental Fig. 1).

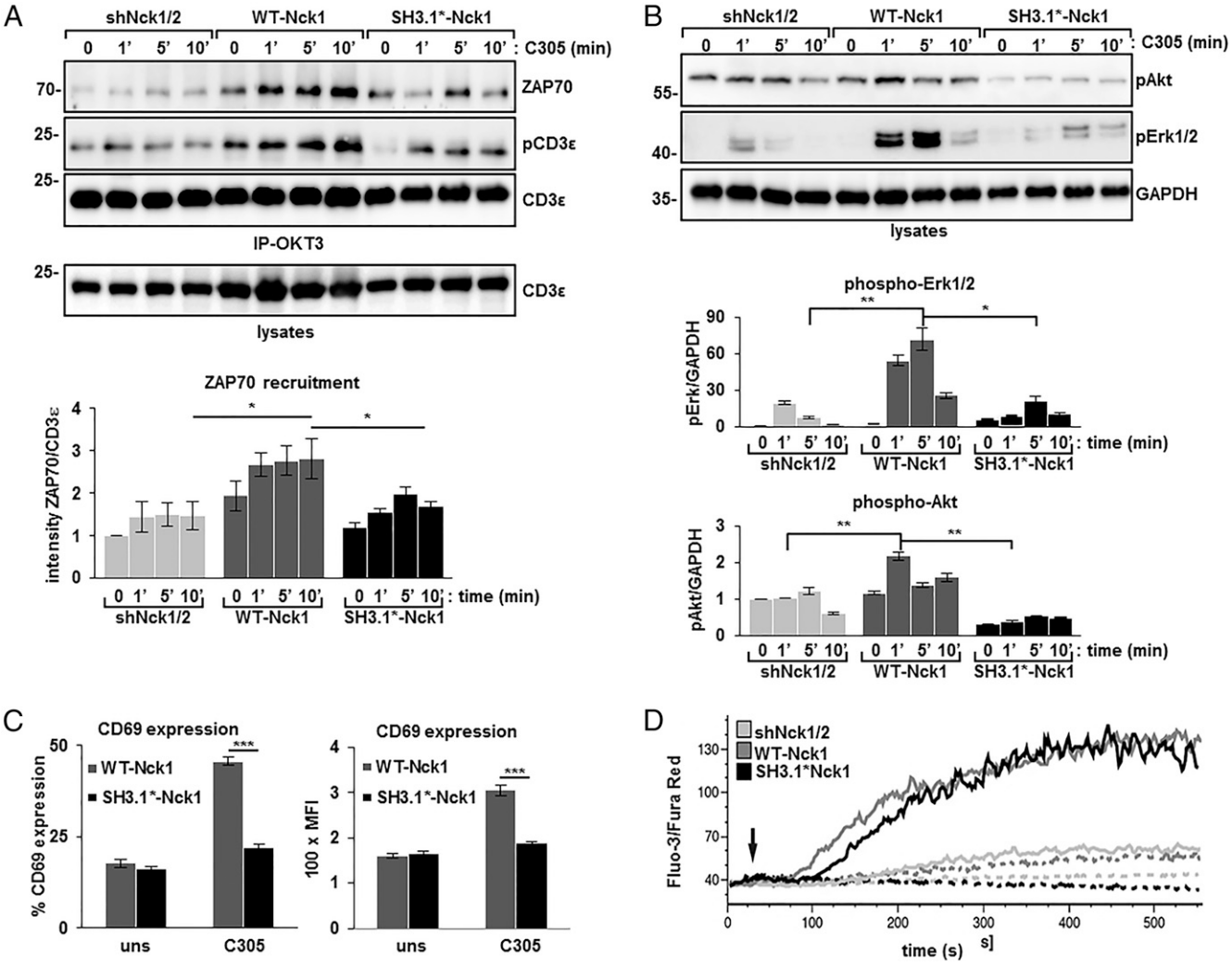

FIGURE 5. Preventing the Nck1–CD3ε association impairs T cell activation. (**A**) shNck1/2 cells stably expressing WT-Nck1 or SH3.1*-Nck1 were stimulated with the anti-TCR Ab C305 for the indicated times. The CD3 immunoprecipitates were immunoblotted with the indicated Abs. The signal intensity of ZAP70 was quantified and presented as a ratio of ZAP70 to CD3ζ normalized to unstimulated shNck1/2 cells (data are representative for three experiments). (**B**) The indicated cells were treated as in (A) and cell lysates were separated by SDS-PAGE and the Western blot developed with anti–phospho-Akt (pAkt) and anti–phospho-Erk1/2 (pErk1/2) Abs. GAPDH serves as a loading control. The quantified signal intensities are presented as a ratio of the phospho-Erk1/2 and phospho-Akt to the corresponding GAPDH values normalized to the value of unstimulated shNck1/2 cells. Data are representative of three experiments (data represent the mean ± SEM). (**C**) WT-Nck1– and SH3.1*-Nck1–expressing shNck1/2 cells were stimulated with C305 for 6 h and stained with an anti-CD69 Ab following flow cytometry quantification. The percentage of $CD69^+$ cells (*left panel*) and their mean fluorescence intensity (MFI, *right panel*) are displayed. Data are representative of four experiments and represent the mean ± SEM. (**D**) shNck1/2-, WT-Nck1–, and SH3.1*-Nck1–expressing cells were either left untreated (dotted line) or treated with anti-TCR Ab C305 (solid line) and Ca^{2+} influx was measured using flow cytometry. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Activation of Erk is required for CD69 expression in T cells (36, 37). In line with this, TCR-induced CD69 expression was absent in the SH3.1*-Nck1–expressing cells, whereas expression of CD69 was induced in the WT-Nck1–expressing cells (Fig. 5C).

Finally, we tested the influence of the SH3.1*-Nck1 mutant on calcium influx. The calcium response was strongly impaired in shNck1/2 cells, indicating that Nck is required for coupling the TCR to calcium influx. However, in the SH3.1*-Nck–expressing cells the same extent of calcium influx was detected as in WT-Nck1–expressing cells (Fig. 5D). This could suggest that strong Nck binding to the TCR is not required for TCR-induced calcium influx.

Taken together, these data indicate that strong Nck recruitment to the TCR is required for some (Akt, Erk1/2, CD69), but not for all (calcium), signaling events downstream of the TCR.

Discussion

The interaction of the recombinant SH3.1 domain of Nck with CD3ε has been studied in detail (13, 18–22). However, the molecular mechanism by which full-length Nck binds to the TCR has not been studied to date, although it has been speculated that recruitment of Nck to the TCR plays an important role for TCR triggering (13, 22, 35). In this study, we show that Nck uses both its SH3.1 and SH2 domains in a cooperative manner to bind to partially phosphorylated CD3ε.

Using the in situ PLA and coimmunopurifications, we found that Nck and the TCR associate with each other upon anti-TCR Ab stimulation. TCR triggering leads to the exposure of the CD3ε PRS to which the SH3.1(Nck) domain can bind (13), providing a molecular explanation for the ligand-induced recruitment of Nck to the TCR. However, we surprisingly found that Nck was not recruited to the TCR upon anti-TCR stimulation when tyrosine phosphorylation was inhibited using the kinase inhibitor PP2 or when Lck-deficient cells were used. Because the active TCR conformation is stabilized by ligand binding also in the presence of PP2 (14, 15, 18), our data suggest that TCR phosphorylation is needed for efficient Nck binding. Furthermore, we found that the Nck–TCR interaction increased within the first 5 min after TCR ligation, correlating with the increase of TCR phosphorylation. Moreover, phosphorylation of the second tyrosine (Y50) of the CD3ε is essential for Nck binding. These data again support the notion that TCR phosphorylation enhances Nck recruitment to the TCR. However, phosphorylation of tyrosine 39 (Y39) within the CD3ε PRS blocks binding of the SH3.1(Nck) domain to the CD3ε PRS (32).

The requirement of TCR phosphorylation for Nck recruitment to the TCR suggested that the SH2(Nck) domain might play a previously unappreciated role. Indeed, we found that the purified SH2(Nck) domain can bind to the cytoplasmic tail of CD3ε, but only when one or both of the CD3ε tyrosines were phosphorylated. Likewise, an intact SH2 domain in full-length Nck was required for a strong association of Nck to the TCR. Thus, the interaction between the SH2(Nck) domain and a phosphotyrosine of CD3ε is required for optimal Nck recruitment to the stimulated TCR.

Next, we induced massive phosphorylation of the TCR using the phosphatase inhibitor PV, which does not stabilize the active TCR conformation and thus does not result in the binding of the purified SH3.1(Nck) domain to the TCR (13, 18). Based on our finding that the SH2(Nck) domain can bind to phosphorylated CD3ε tyrosines, we expected to detect Nck binding to the TCR upon PV stimulation. However, PV did not induce Nck recruitment to the TCR. Furthermore, PV treatment prevented Nck association when combined with anti-TCR Ab stimulation. Thus, it seems that massive phosphorylation of the TCR abolishes Nck recruitment, being in line with the finding that phosphorylation of the CD3ε residue Y39 within the PRS abrogates SH3.1(Nck) binding (32). Indeed, NMR structures of the PRS binding to SH3.1(Nck) revealed that the nonphosphorylated Y39 is a crucial interaction point with the hydrophobic pocket of SH3.1(Nck) (33, 34). Indeed, the Nck–TCR interaction decreased at 10 min after TCR ligation, correlating with very strong CD3ε phosphorylation.

As discussed, the PV data suggested that the SH3.1(Nck) domain might interact with the nonphosphorylated and exposed CD3ε PRS. Indeed, we and others (13, 18) found that the purified SH3.1(Nck) domain can bind to the cytoplasmic tail of CD3ε, but only when Y39 was not phosphorylated. Likewise, an intact SH3.1 domain in full-length Nck was required for a stable association of Nck to the TCR. Thus, the interaction between the SH3.1(Nck) domain and the nonphosphorylated PRS is required for Nck recruitment to the stimulated TCR.

To resolve our seemingly paradoxical findings, we used synthetic CD3ε cytoplasmic tail peptides to mimic unphosphorylated and different patterns of phosphorylated CD3ε. Full-length Nck could only strongly associate to the peptide where the first tyrosine (Y39) was not phosphorylated, thus allowing the SH3.1(Nck) domain to bind, and when the second tyrosine (Y50) was phosphorylated, thus promoting SH2(Nck) domain binding. Because the interaction with one domain alone was not sufficient for optimal Nck binding, we conclude that both interactions have to occur in a collaborative manner (Fig. 6). Therefore, the exact pattern of phosphorylation of CD3ε determines Nck recruitment and could thus specify downstream signaling. It has been shown that agonistic stimulation leads to a different pattern of CD3ζ phosphorylation than antagonistic stimulation (38). However, whether this different pattern causes differentially downstream signaling is not yet resolved (39).

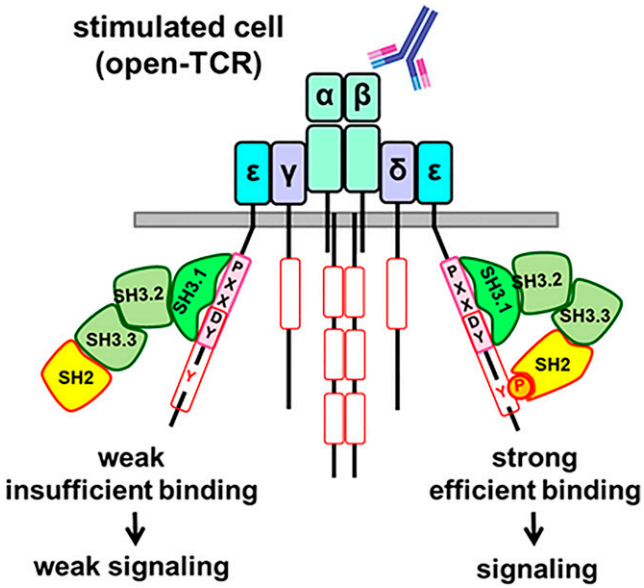

FIGURE 6. Illustration of Nck-binding to the ligand-/Ab-engaged TCR. Triggering of the TCR induces a conformational change of CD3ε (active TCR conformation), resulting in the exposure of the PRS (pink boxes). The exposed PRS can consequently interact with the SH3.1 domain of Nck (*left*), but only when the tyrosine contained in the PRS is not phosphorylated. This binding mode is suboptimal, thus promoting weak TCR signaling (*left*). If the second ITAM tyrosine of CD3ε is phosphorylated in the active TCR, Nck can bind with its SH3.1 and SH2 domains in a cooperative manner (*right*). This binding mode is strong, allowing optimal recruitment of Nck to the TCR promoting TCR signaling (*right*).

Although the endogenous Nck needs both the SH3.1 and the SH2 domains to optimally bind to the TCR, the purified SH3.1(Nck) domain binds with sufficient avidity to be used in a pull-down assay to purify the TCR in its active conformation (13, 15). According to the law of mass action, we suggest that the huge excess of SH3.1 (Nck), as used in the pull-down assays, shifts the equilibrium toward TCR binding. The same might hold true for the SH2(Nck) domain under similar conditions. An alternative interpretation would be that Nck exists in an autoinhibited state, in which the SH3.1 domain cannot bind to the TCR and SH2 binding would free the SH3.1 domain. The isolated SH3.1 domain would always be available for binding to the CD3ε PRS.

Although the exact architecture of the TCR complex is unknown, some studies suggest that the CD3 heterodimers are clustered on one side of the TCR (40). Specifically, the two CD3ε chains seem to be juxtaposed to each other. We demonstrated that one Nck molecule can simultaneously interact with two CD3ε cytoplasmic tail peptides, one providing the SH3.1(Nck) interaction and the other one the SH2(Nck) interaction. However, phosphorylated CD3γ and CD3δ peptides cannot substitute for the phosphorylated CD3ε peptide, suggesting that the CD3ε subunit is the optimal binding site for Nck and that two Nck molecules can potentially simultaneously bind to the TCR.

Nck has been discussed to play a role at the TCR by binding to CD3ε (13, 22, 35) and at SLP76 by binding to phosphorylated SLP76 using its SH2(Nck) domain (10–12). Additionally, the SH3.3(Nck) domain binds to WASp, thus recruiting WASp to the SLP76 signalosome, promoting actin polymerization (11, 41, 42). Because Nck binds to WASp with its SH3.3(Nck) domain (42), it is possible that Nck serves as a physical bridge between the TCR and WASp (43), regulating cytoskeleton dynamics from the TCR.

In the present study, we used the shNck1/2 cells to express Nck1 molecules in which only the SH3.1 domain was mutated, and thus Nck was not able to optimally bind to the TCR. We found that after TCR engagement, CD3 was phosphorylated less in shNck1/2 and SH3.1*-Nck cells as compared with WT-Nck cells. This was associated with a decrease of ZAP70 recruitment to the TCR in the shNck1/2 and SH3.1*-Nck cells. Thus, Nckbinding to the TCR plays a crucial role by promoting CD3 phosphorylation. These findings are in agreement with a study using knock-in TCR transgenic mice bearing a CD3ε PRS mutation (22). Additionally, we showed that phosphorylation of Erk and expression of CD69 were strongly reduced in shNck1/2 and SH3.1*-Nck1 cells, as expected from reduced CD3 phosphorylation.

Furthermore, TCR-induced Ca^{2+} influx was defective in shNck1/2 cells but restored in the SH3.1*-Nck1–expressing cells. This could indicate that Nck is required for Ca^{2+} mobilization independent of its TCR binding capability. However, because CD3 phosphorylation is upstream of Ca^{2+} influx (44), we suggest that residual CD3 phosphorylation might be sufficient to promote Ca^{2+} signaling in our setting. This is in line with studies where mutation of the CD3ε PRS still allowed TCR-induced Ca^{2+} influx (45), but not with others where Ca^{2+} signaling was drastically reduced upon prevention of the CD3ε–Nck interaction (17, 46). This discrepancy might be related to the different mutations used. In conclusion, it seems that the level or the pattern of TCR phosphorylation needed for induction of Ca^{2+} influx is quantitatively or/and qualitatively different from the one needed to induce Erk or AKT phosphorylation.

We speculate that phosphorylation of the two CD3ε tyrosines is differentially regulated in time (Y50 is phosphorylated earlier than Y39), so that Nck is initially recruited to the TCR, promoting TCR activation, but dissociates after longer stimulation times to qualitatively and/or quantitatively alter TCR signaling. Thus, CD3 tyrosines are not redundant and might have specific roles. The implications need to be explored.

In summary, in the present study we identify the molecular requirements for Nck binding to the TCR, highlighting that the exact pattern of CD3 phosphorylation (in this case a partial CD3ε phosphorylation) might be critical for TCR functioning and T cell activation (Fig. 6). Thus, Nck is an important adaptor protein that participates in the initiation of TCR signaling at the TCR.

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Disclosures

The authors have no financial conflicts of interest.

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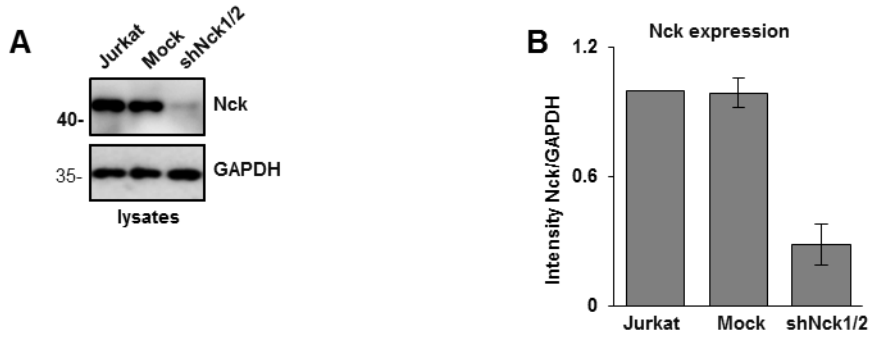
Figure S1 Nck expression in shNck1/2 cells



Supplemental Figure S1.

(A) Jurkat T cells were either transfected with a control vector (mock) or Nck1 and Nck2-specific shRNA expression vector (shNck1/2). Single clones were selected by limiting dilution technique. Individual clones were analyzed the expression of Nck protein using immunoblotting with anti-Nck1 and anti-GAPDH antibodies as a loading control. A single clone with the most down-regulated Nck1/2 expression was selected for further study. **(B)** The quantified signal intensities are presented as a ratio of Nck to the corresponding GAPDH values normalized to the value of Jurkat cells. Data are representative of three experiments. The mean ± SEM is shown.