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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 sequences (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 (CD3ε, CD3γε) and a ζζ dimer, which are responsible for signal transduction (4, 5). The TCR bears 10 ITAMs (YXXI/I–X 6–8, YXXL/I) (6), a single ITAM in each CD3ε, CD3γ, and CD3ζ, 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 initiating signaling events, as for example Ca2+ 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
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 interaction 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 (Y191) Abs were purchased from Cell Signaling Technology; the mouse anti-CD3ε Ab (C305) 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-CD3ε Ab (pY185) and the inhibitor of Src family kinases PP2 were from Sigma-Aldrich (St. Louis, MO). The anti-β-actin antiserum 448 and the anti-phospho-CD3ε antisera (anti-phospho-εY1) have been described (24, 25). The below-mentioned peptides were obtained from Eurogentec: pepPP, YWSKNRKANKVTRGAGSRPRGQNKERPPPVPNPD; pepPP, AGQDGVRQSRASDKQTLLQNEQLPP2 (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 TRITC 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.

In vitro proximity ligation assay

Jarkut or primary CD4ε 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 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. To induce CD69 expression, 2 ng/ml hygromycin. Nck1 expression was assessed by the Duolink kit (Olink Bioscience), which provides resistance to hygromycin, and for further studies, clones with similar expression levels were selected.

Cell cycle, cell viability, and apoptosis

For the CA3ε 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 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. FlowJo 6.1 software was used for data analysis.

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CA3ε 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 Fura Red (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. FlowJo 6.1 software was used for data analysis.
Src kinase activity for Nck binding to the TCR was also observed due to low levels of Lck activity or to binding of the SH3.1 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 proximity was observed 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 communoprecipitated 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 communoprecipitation 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 communopurification 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 PFS 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 Nck–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 (p39ε), the doubly phosphorylated CD3ε tail (pp50ε), 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 biotinylated CD3ε peptides (p39ε, p50ε, and ppe), 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 PFS 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–PFS (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 phosphorylatable tyrosines, we test
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 located on different CD3ε molecules. To this end, Jurkat lysates...
Because we found that partial CD3ε requires the SH3.1 and SH2 domains to bind to the TCR. Hence, we hypothesized that Nck might use its SH3.1 and SH2 domains to bind to the TCR. Nck1 mutant did not bind strongly to any peptide, indicating that the SH3.1 domain is crucial for Nck binding to the TCR. 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. The SH3.1*-Nck1 protein was expressed to a similar level as the WT-Nck1 protein, and the expression level of both proteins was similar amounts of TCR on their cell surface as shown by flow cytometry (Fig. 4C).

SH3.1(Nck) AND SH2(Nck) 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. Nck requires the SH3.1 and SH2 domains to bind to the TCR. Nck1 mutant did not bind strongly to any peptide, indicating that the SH3.1 domain is crucial for Nck binding to the TCR.

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. Nck from the cell lysates bound better to the p50ε peptide and a weaker binding to the nonphosphorylated peptide, than to the sum of Nck binding to the p50ε peptide, which cannot mediate SH2 binding (Fig. 3E). 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 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.

Next, we repeated this experiment using the SH2*-Nck1– 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 CD3ε peptide (p50ε or p50β), 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 ppe than to ε alone or ppe alone (Fig. 3E). Importantly, there was a synergistic effect, in that more Nck bound to ε and ppe than the sum of Nck binding to ε alone and ppe alone. This synergistic effect was not observed when ε was mixed with pγ or pδ (Fig. 3D). Collectively, these data suggest that Nck is able to interact with juxtaposed CD3ε, with one being nonphosphorylated and one being phosphorylated.
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). 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

FIGURE 3. Only partially phosphorylated 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. Bead-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 CD3δ peptides as indicated. Bead-bound proteins and the lysates as controls were detected as in (B). *p < 0.05, **p < 0.01.
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 non-functional 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 immuno-blotted 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 Ca2+ influx was measured using flow cytometry. *p < 0.05, **p < 0.01, ***p < 0.001.

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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 the TCR. However, phosphorylation of tyrosine 39 (Y39) within the CD3ε cytoplasmic tail peptides to mimic unphosphorylated and 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 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 CD3ε 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). The SH3.3(Nck) domain binds to WASp, thus recruiting WASp to the TCR. SLP76 signalosome, promoting actin polymerization (11, 41, 42). The SH3.3(Nck) domain binds to WASp, thus recruiting WASp to the TCR. SLP76 signalosome, promoting actin polymerization (11, 41, 42).

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, CD3δ was phosphorylated less in shNck1/2 and SH3.1*-Nck cells as compared with WT-Nck cells. This was as- 

In summary, in the present study we identify the molecular requirements for Nck binding to the TCR, highlighting that the exact pattern of CD3δ 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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References


**Corrections**


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