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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 adaptocatalytic tyrosine kinase (Nck) can bind to the exposed PRS of CD3e, 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, and it is based on two cooperating interactions. First, the SH3.1(Nck) domain has to bind to the nonphosphorylated 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.
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-CD3ε (OKT-3) Ab was from eBioscience; the anti-idiotypic TCR Ab (C305) was from Millipore; the anti-ZAP70 and goat anti-CD3ε Abs (Sigma-Aldrich) using the following primers: 5′-GCTGCTAATTTCTCGGAGAGGG-3′ (M-4000; GE Healthcare Life Sciences). Band intensity was assessed by the ImageQuant LAS 4000 (GE Healthcare). 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 ligase assay

Juratk and 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% Triton X-100, and stained with an allophycocyanin (Ab M20 [Santa Cruz Biotechnology]) and a rabbit anti–phospho-threonine Tyr142 Ab (Cell Signaling Technology) at 37°C for 6 h and then stained with an allophycocyanin-conjugated anti-CD69 Ab. The percentage of CD69+ cells was measured by FACSCalibur (BD Biosciences). Quantification 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 × 10^6 cells were starved overnight in a medium containing 1 mM NaF 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 measured 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 to the TCR 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 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 PLAs 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 coimmunoprecipitation 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 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), and the doubly phosphorylated CD3ε peptide (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 phosphorylatable tyrosines, we test...
whether the SH2(Nck) domain might be sufficient to bind full-length Nck to CD3ε 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 CD3ε and CD3δ (Fig. 3D). We found that Nck could not bind to CD3ε 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 phosphotyrosine can be located on different CD3ε molecules. To this end, Jurkat lysates
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 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 Fig. 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-ξ Abs. The lysates were developed with anti-ξ. Data are representative of five experiments, and the statistical analysis was done as in Fig. 3 (Fig. 4B). (C) Primary human CD4+ T cells were purified fromuffy 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.
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 phosphorylated 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-CD3ε Ab (OKT3) following immunoblotting with anti-Nck1, anti-pCD3ε, and anti-ξ 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).
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 CD3e.

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 SH2(Nck) domain and the nonphosphorylated PRS is required for 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 CD3ε phosphorylation than antagonistic stimulation (38). However, whether this different pattern causes differentially downstream signaling is not yet resolved (39).
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 CD3ζ 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 signalsome, 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 Ca²⁺ influx was defective in shNck1/2 cells but restored in the SH3.1*-Nck1-expressing cells. This could indicate that Nck is required for Ca²⁺ mobilization independent of its TCR binding capability. However, because CD3 phosphorylation is upstream of Ca²⁺ influx (44), we suggest that residual CD3 phosphorylation might be sufficient to promote Ca²⁺ signaling in our setting. This is in line with studies where mutation of the CD3ε PRS still allowed TCR-induced Ca²⁺ influx (45), but not with others where Ca²⁺ 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 Ca²⁺ 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

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References


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Corrections


A source of funding was omitted in this article as originally published. The corrected funding information footnote should read: “P.P. and P.W. were supported by the Royal Golden Jubilee Ph.D. Programme of the Thailand Research Fund. S.P. has received support from Naresuan University Grant R2558B105 and by Thailand Research Fund Grant RSA5880009. This work was also supported by German Research Foundation Grant EXC294 (BIOSs) (to O.S.Y., S.M., and W.W.S.), European Union Grant FP7/2007-2013 (SYBILLA) (to W.W.S. and E.P.D.), an Innovationfonds Forschung 2012 grant (to F.A.H. and S.M.), and by the Excellence Initiative of the German Research Foundation (GSC-4, Spemann Graduate School).”

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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.