Role of Nck in T Cell Receptor Activation

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Summary

The non-catalytic region of tyrosine kinase (Nck) is an important regulator of the cytoskeletal reorganization and of the formation of the immune synapse between T cells and antigen presenting cells (APCs). Nck acts as a link between the TCR:CD3 complex and signaling molecules that mediate cytoskeleton rearrangement and T cell activation. The intracellular T cell signaling both in an immunoreceptor tyrosine-based activation motif (ITAM)-requiring mechanism and in a non-ITAM-requiring mechanism may be regulated by Nck. Therefore, this review attempts to summarise the critical role of Nck in T cell receptor activation. The subsequent applications may contribute to the usage of Nck as a target for pharmacological modulation of the immunopathologic conditions.

Keywords:Nck, adaptor protein, TCR activation, T cells

Introduction

T cells are thekey cellular constituents of the immune system that possess the capacity todirectly destroy cells infected with a pathogen and have a central role in stimulating and coordinating withother components of the immune response, such as B cells.The TCR:CD3 surface expression is essential for T cell development and function and is therefore a key element in the initiation of the adaptive immune response (Dave, et al., 1997; Haks, Krimpenfort, Borst, & Kruisbeek, 1998; Love, et al., 1993; Wang, et al.,1999). The TCR:CD3 complex recognizes a foreign peptide presented by a major histocompatibility (MHC) molecule. Binding of TCR:CD3 complex to peptide-MHC complex molecule initiates a specific immune response. T cell signaling is transduced not by the TCR itself but by invariant proteins called CD3 and ζζ within the TCR complex. The ITAMs, which are located in the cytoplasmic domains of CD3 and ζchains are phosphorylated. This results in activation of downstream signaling molecules and initiation of signaling complexes (Lin, & Weiss, 2001; Rudolp, & Wilson, 2002; Werlen, & Palmer, 2002).

Numerous proteins and protein complexes constitute the T cell signaling pathway. Signaling pathways usually start with the alteration of intracellular molecules of receptor proteins that create various signal transduction cascades. The signaling molecules, which regulate the formation of multimeric protein complexes, can be divided into three groups: (1) enzymes, such as Src family tyrosine kinases, phospholipase Cγ and RasGTPase-activating protein (RasGAP), (2) regulator molecules, such as the Vav family of Rho guanine nucleotide exchange proteins, Cbl and signal transducer and activator of transcription (STAT) proteins, and (3) adaptor proteins without any known enzymatic activities (Buday, Wunderlich, & Tamas, 2002). Adaptor proteins usually contain several domains within their structures (e.g., Src homology 2

(SH2) and SH3 domains), allowing specific interactions with several other specific proteins. These proteins mediate specific protein-protein interactions that drive the formation of protein complexes for the activation of signal transduction cascades inside the cell. This review will focus on a role of Nck that is important for T cell activation and emphasize the various ways in which this adaptor carries out its essential functions.

The Nck Family of Adaptor Proteins

Nck (non-catalytic region of tyrosine kinase) is a cytosolic adaptor molecule of 47-kDa. Nck is expressed in a wide variety of cells and tissues. The human NckcDNA was originally cloned by Lehmann, Riethmuller, and Johnson (Lehmann, Riethmuller, & Johnson, 1990). Using monoclonal antibodies recognizing the melanoma specific MUC18 antigen, Nck was identified as a false positive during the screening of a melanoma cDNA expression library. Nck has been cloned for the second time by virtue of its ability to bind the phosphorylated C-terminal tail of the epidermal growth factor (EGF)receptor in the cloning of receptor targets (CORT) screening technique (Skolnik, et al., 1991). The murine homolog of Nck, termed Grb4 was later cloned in an attempt to identify novel SH2 domain containing proteins (Margolis, et al., 1992). In human cells, the Nck family comprises two highly related members (Nck1/Nckα and Nck2/Nckβ, also termed Grb4). Nck1 and Nck2 display 68% identity of overall amino acid residues. Notably, the largest differences are mainly located in the linker regions between the interaction modules (Figure 1). While the human nck-1 gene has been localized to the 3q21 locus of chromosome 3, the *nck-2* gene can be found on chromosome 2 at the 2q12 locus (Huebner, et al., 1994; Vorobieva, et al., 1995). However, hardly any Nck1- or Nck2-specific downstream target has been identified so far. In fact, in many instances the interactions have not been clearly attributed to Nck1 or Nck2. Therefore, Nck1 and Nck2 are generally termed Nck. Nck is a prototype of SH domain-containing adaptor proteins that facilitate multiple interactions with other related proteins and form functional signaling complexes. Nck molecule consists of a single C-terminal SH2 domain, which binds to phosphotyrosine residues, and three N-terminal SH3 domains, which bind to proline-rich sequence (PRS) within specific peptide sequence contexts of proteins. Nck has been reported to be able to interact with approximately 60 different proteins, suggesting that it is used by a wide variety of intracellular signaling pathways (Buday, et al., 2002).

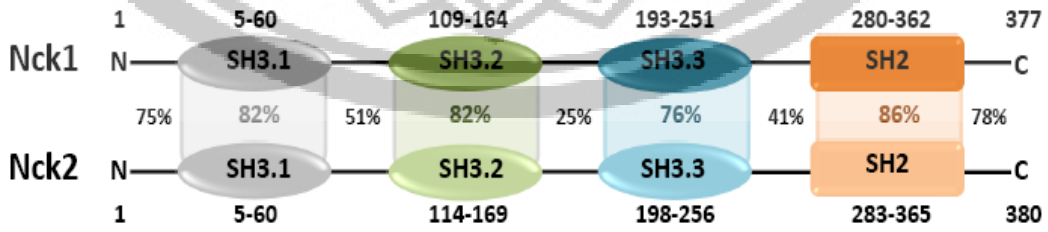

Figure 1 Diagram illustrating the architecture of Nck family

Figure 1 Diagram illustrating the architecture of Nck family. (A) Nck1 and Nck2 contain identical domains, which are three Src Homology 3 (SH3) domains at N-terminus and a Src Homology 2 (SH2)domain at C-terminus. The percent similarity of amino acids of the domains and linker regions between Nck1 and Nck2 are shown. The numbers of amino acid length of Nck1 and Nck2 are shown at the C-terminus.

Nck can bind to a number of phosphotyrosine proteins, such as activated EGF and platelet-derived growth factor (PDGF) receptor tyrosine kinases (Chou, Fajardo, & Hanafusa, 1992; Li, Hu, Skolnik, Ullrich, & Schlessinger, 1992; Meisenhelder, & Hunter,1992), downstream of tyrosine kinases (Dok) (Noguchi, et al., 1999), and SH2-domain-containing leukocyte protein of 76 kDa (SLP-76) (Wunderlich, Farago, Downward, & Buday, 1999) via its SH2 domain. SH2 domain links the Nck proteins to upstream tyrosine-phosphorylated receptor and cytosolic proteins to downstream signaling pathways. To date, there are more than twenty tyrosine-phosphorylated proteins that can interact with SH2 domains of Nck1 and Nck2 (Lettau, Pieper, & Janssen, 2009). Although SH2domains of both Nck isoforms can recognize the consensusmotif of pYDxV (AYST) x (DEC) (when x is any amino acid) on the partner proteins, severalpieces of evidence have indicated some non-overlapping functions of SH2 domains between Nck1 and Nck2 (Chen, She, Kim, Woodley, & Li, 2000; Lettau, et al., 2009; Nishimura, et al., 1993; Pramatarova, et al., 2003). Upon TCR triggering, SH2 domain of Nck is recruited to phosphorylated SLP-76 via two tyrosine-phosphorylated sites, Tyr113 (YESP) and Tyr128 (YESP) (Wunderlich, et al., 1999).

SH3 domains of Nck mediate the interaction with their downstream targets proteins, which contain PRS (Lettau, et al., 2009). Early biochemical studies have identified several proteins that can interact with one or more of the Nck SH3 domains. Those proteins are, for examples, son of sevenless (Sos) (Hu, Milfay, & Williams, 1995; Okada, & Pessin, 1996), Vav (Ramos-Morales, Druker, & Fischer, 1994), Wiskott-Aldrich syndrome protein (WASP) (Rivero-Lezcano, Marcilla, Sameshima, & Robbins, 1995), Nck-associated p21-activated kinase 1 (PAK1) (Bokoch, et al., 1996) and CD3ε (Gil, Schamel, Montoya, Sanchez-Madrid, & Alarcon, 2002). To date, over thirty proteins have been reported to interact with SH3 domains of Nck1 and Nck2. Moreover, several Nck ligands bind to more than one SH3 domain of Nck. This suggests that a cooperative interaction is necessary for tight complex formation (Wunderlich, Goher, Farago, Downward, & Buday, 1999). Nck utilizes the specificity of its individual SH3 domains to facilitate multiple interactions with different associated proteins.

Nck can recruit proline-rich proteins to the plasma membrane. The proline-rich proteins are also recruited by Nck to the multiple protein complexes found either in the cytoplasm or in association with the actin cytoskeleton. It has been reported that Nck is involved in many aspects of tissue development, immune cell activation and tumorigenesis. These processes, which depend on changes in cell polarity, morphology and migration, are regulated by protein complexes formed by Nck (Buday, et al., 2002; Lettau, et al., 2009). It has been reported that Nck acts as a link between extracellular and intracellular signaling molecules as well as cytoskeleton (Lehmann, et al., 1990; Park, 1997; Ullrich, & Schlessinger, 1990). In T cells, Nck plays a pivotal role in the TCR-induced reorganisation of the actin cytoskeleton and the formation of the immunological synapse between T cells and antigen presenting cells (APCs). Two different models for the association of Nck with TCR/CD3 complex have been proposed. In an ITAM-requiring pathway, Nck is recruited to the TCR complex via phosphorylated SLP-76

(Wunderlich, et al., 1999), another central constituent of the membrane proximal activation complex. In a non-ITAM-requiring pathway, dependent on an activation-induced conformational change in the CD3ε subunits, a direct binding of Nck to components of the TCR/CD3 complex has been reported (Gil, et al., 2002). In this regard, activation-dependent association between Nck and CD3ε PRS occurs prior to ITAM phosphorylation.

Besides Nck, there are a number of SH2/SH3 adaptor proteins that link upstream signal from TCR, through their SH2 domains to different downstream signaling pathways, through their SH3 domains. These proteins are Crk, Grb2 (growth factor receptor-bound protein 2), Gads (Grb2-related adaptor downstream of Shc), and p85. There functions in T cell activation are discussed briefly. Crk (Crk-II and Crk-L) consists of a single SH2 domains and two SH3 domains located at N- to C-terminus, respectively, in order SH2-SH3-SH3 (Lin, & Weiss, 2014). Crk is associated with phosphorylated Cbl via its SH2 domain upon TCR triggering, which is required for TCR-regulated integrin adhesion (Nolz, et al., 2008). Grb2 is built of an SH2 domain and two SH3 domains, in order SH3-SH2-SH3. The Grb2 SH3 domain constitutively interacts with SOS and also upon TCR stimulation. This Grb2/SOS interaction is required for TCR-induced Ras-Erk signaling cascade (Smith-Garvin, Koretzky, & Jordan, 2009). Gads is an SH3-SH2-SH3 adaptor protein implicated in the linking of phosphorylated LAT, through Gads SH2 domain, to SLP76, through Gads PRS upon TCR stimulation. The formation of this complex is essential for the activation of various signaling pathways such as MAPK and Ca2+ pathways (Yoder, et al., 2001). The p85 is the SH3-SH2-SH2 adaptor protein, which is a regulatory subunit of PI3K (phosphatidylinositol 3-kinase) (McCarty, 1998). Following TCR engagement, the p85 SH2 domain mediates the localization of PI3K to plasma membrane, where PI3K is activated and controls the transcription of cytokine genes (Kang, Schneider, & Rudd, 2002).

Thus, it is possible that the recruitment of Nck to CD3ε interferes with subsequent phosphorylation of the CD3εITAM by Src kinases. To date, the function of Nck in T cell receptor signaling has not been completely understood.

Nck and T Cell Effector Function

Actin Reorganization in T Cell Activation

Actin cytoskeleton consists of a network of filaments, which is composed of individual actin protein. There are two forms of actin protein in cells, a monomeric form (globular or G-actin) and a polymerized form (filamentous or F-actin) (Blasutig, 2008). TCR activation triggers actin dynamics for controlling vital T cell responses, such as T cell adhesion, motility, and proliferation. These depend on the recruitment of Nck and WASP to the site of TCR activation. Upon optimal TCR engagement, ITAMs are phosphorylated, resulting in activation of zeta-chain (TCR) associated protein kinase 70 kDa (Zap70). Activated Zap70 then phosphorylates linker for activation of T cells (LAT) and SLP-76. The phosphorylated SLP-76 is the site for recruitment of SH2 containing proteins. These are Nck, Vav1, and the nucleation-promoting factors (NPFs) including the WASP, WASP-family verprolin-homologous protein-2 (WAVE2) and hematopoietic cell lineage-specific protein 1 (HS1) (Wu, & Koretzky, 2004).

Since Nck has no intrinsic ability to mediate actin polymerization, Nck acts as a scaffold protein to recruit other signaling proteins to form signaling complexes that can induce actin polymerization. Nck

can interact with phosphorylated SLP-76 through its SH2 domain and can associate with PAK, Vav1, and WASP (through its SH3 domains Bubeck, et al., 1998; Bunnell, et al., 2002: Fischer, et al., 1998; Galisteo, Chernoff, Su, Skolnik, & Schlessinger, 1996; Rivero-Lezcano, et al., 1995; Wunderlich, et al., 1999).

The main function of the multidomain adapter protein WASP is the activation of the actin-related proteins 2 and 3 (Arp2/3) complex. Arp2/3 is the seven-subunit complex that is in an inactive conformation in resting state, but can be activated by WASP and WAVE2 upon T cell stimulation. The recruitment of Arp2/3 to WASP coupled with actin monomer induces the formation of branched actin filament networks (Figure 2) (Panchal, Kaiser, Torres, Pollard, & Rosen, 2003). Nck not only passively interacts with WASP and may thus recruit WASP to molecular activation clusters (Rivero-Lezcano, et al., 1995), but also modulates WASP activity (Rohatgi, Nollau, Ho, Kirschner, & Mayer, 2001). Therefore, this adapter protein also regulates activation-dependent reorganization of TCR-associated signaling complexes and platforms.

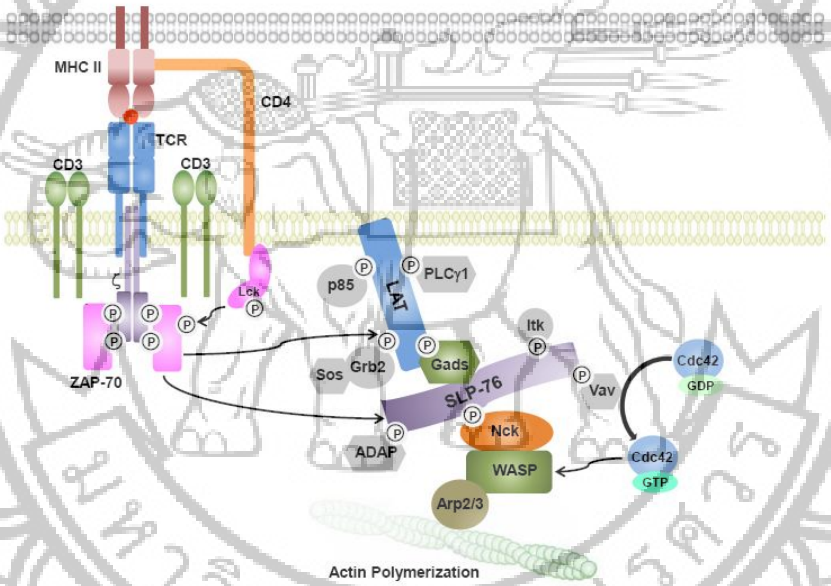

Figure 2 Schematic diagram of molecular signaling cascades that link the TCR signaling to cytoskeleton rearrangement

Figure 2 Schematic diagram of molecular signaling cascades that link the TCR signaling to cytoskeleton rearrangement. After TCR ligation with its ligand, antigenic peptide bound on the MHC molecule that is expressesed on the antigen presenting cells (APCs) surfaces. This leads to the phosphorylation of ITAMs located at the cytoplasmic tails of CD3 and ζ molecules by protein tyrosine kinase Lck that is associated with the cytoplasmic tail of CD4 molecule. ZAP-70 is then recruited to the phosphorylated ITAMs, where it is activated. The activated ZAP-70 in turn phosphorylates LAT and SLP-76. The phosphorylated LAT serves as docking sites for adaptor proteins such as Grb2 and Gads. Subsequently, SLP-76 is recruited to LAT-nucleated complex via Gads. Like LAT, SLP-76 is an adaptor protein that contains the sites for interaction with various proteins such as Nck, Vav, Itk and ADAP. Nck then recruits the adapter protein WASP. The GEF Vav promotes the exchange of Cdc42-GDP to

Cdc42-GTP that is essential for WASP activation. WASP activates the Arp2/3 complex, which then induces the formation of branced actin filament networks.

Nck and signaling within T cells

Many models have been proposed for how TCR signaling is initiated, including TCR complex conformational change. Conformational change in the TCR complex upon antibody binding has been proposed to explain studies of T cell signaling where receptor multimerization or the avidity of antibodies for the receptor do not sufficiently explain the biochemical results (Janeway, 1995). This idea is attractive because the ITAMs in the cytoplasmic domains of these components are the proximal targets of tyrosine phosphorylation. Tyrosine residues within these motifs, once phosphorylated by the Src family tyrosine kinase (SFK), become binding sites for different SH2-containing proteins, including the tyrosine kinase ZAP70 and then initiate a cascade of downstream signaling events that result in nuclear transcriptional changes as well as cellular morphological changes (Lin, & Weiss, 2001). Several studies have indicated that activated Zap70 promotes recruitment and phosphorylation of downstream signaling molecules including SLP-76, Vav1, a Rho family exchange factor, and Nck (Alberola-Ila, Takaki, Kerner, & Perlmutte, 1997; Bubeck, et al., 1998; Galisteo, et al., 1996; Lin, & Weiss, 2001). The assembly of this multiprotein complex regulates target effectors that interact through Nck, including PAK1. Consistent with this model, TCR-dependent activation of PAK1 and inducible association of PAK1 with Nck have been reported. PAK1 is required for the activation of nuclear factor of activated T-cells (NFAT). Prevention of Nck to interact specifically with PAK1 by the expression of a dominant negative allele of Nck containing a W143K mutation at the second SH3 domain inhibits TCR-mediated NFAT activation. Moreover, PAK1 activation is not necessary for TCR-dependent c-Jun N-terminal kinase (JNK) activation, one component of transcription factor activating protein-1 (AP-1) activation pathway. These results indicate that Nck-PAK1 interaction is required for TCR-mediated NFAT activation, but not for JNK activation (Yablonski, Kane, Qian, & Weiss,1998). Another current model for TCR signaling is a non-ITAM-requiring mechanism. In this regard, CD3ε cytoplasmic tail undergoes a conformational change that exposes the PRS for Nck binding upon multivalent ligands-mediated TCR triggering (Gil, et al., 2002; Gil, Schrum, Alarcon, & Palmer, 2005). This association is mediated by the first SH3 domain of Nck. The exposure of TCR transgenic mouse T cells to antigen-pulsed presenting cells has been shown to promote Nck binding to TCR/CD3 complex. Nevertheless, an Nck-CD3ε interaction-inducing antibody, namely OKT3, can induce both Interleukin-2 (IL-2) release and CD69 expression in Jurkat cells (Gil, et al., 2002). Moreover, Nck recruitment to CD3ε occurs earlier than phosphorylation of the CD3ε ITAM and is independent of tyrosine kinase activity. In addition, disruption of the Nck/CD3ε interaction inhibits immunological synapse maturing and T cell activation. Through a cascade of T cell activation, T cells must efficiently recognize APCs or target cells via several complex cytoskeleton-dependent processes, including integrin-mediated adhesion, immunological-synapse formation, cellular polarization, and receptor signaling. The dynamic actin reorganization is required to coordinate these processes and ultimately controls T-cell recognition and activation. Therefore, the direct recruitment of Nck and associated actin regulatory proteins such as

WASP, WIP or PAK1 to CD3ε of TCR might exhibit an alternative means to a direct link between T cell activation to the cytoskeletal reorganization.

The protease-sensitivity assay has revealed that the cytoplasmic tails of CD3ε subunit adopt a compact structure in the TCR triggering and conformational change is transmitted to the tails of CD3ε subunits. In non-stimulated cells, the CD3 subunits are in a closed conformation, marking the PRS and preventing its interaction with Nck (Risueno, Schamel, & Alarcon,2008). These would account for the ability of Nck to bind to CD3ε in resting T cells. However, because Nck and some of its binding proteins, including SLP76, are tyrosine phosphorylated (Buday, 1999; Lin, & Weiss, 2001), it is proposed that the conformational change that promotes Nck recruitment and the activation of phosphotyrosine kinases as a consequence of TCR-CD3 cross-linking soon come together into a common activation pathway (Gil, et al., 2005).

A recent study in mice has shown that Nck-defective T cells fail to proliferate and to produce IL-2 upon stimulation with anti-CD3ε antibody but not with the DAG mimetic phorbol 12-myristate 13-acetate (PMA) and ionomycin (Roy, et al., 2010). The unmodified response of Nck-deficient T cells to PMA and ionomycin stimulation is due to signaling pathway that bypasses the TCR signaling apparatus. PMA induces a PKCs-mediated oxidative signal and RasGRP activation, whereas ionomycin induces intracellular calcium mobilization. Nck deletion also impairs TCR-mediated calcium mobilization and ERK phosphorylation in activated T cells. These results suggest the involvement of Nck in proximal TCR signaling. Our recent studies have also shown that Nck1 is important in TCR/CD3-mediated activation involving the extracellular-signal-regulated kinase (Erk) phosphorylation pathway, as evidenced by the inhibition of T cell function, particularly to an impairment CD69 expression and IL-2 production when its expression is down-regulated (Yiemwattana, et al., 2012). Furthermore, down-regulation of Nck1, but not Nck2, impairs TCR-induced phosphorylation of the mitogen-activated protein kinase kinase (MEK), activation of the AP-1 and NFAT transcription factors and subsequently, IL-2 and CD69 expression (Ngoenkam, et al., 2014). In this scenario, Nck1 could bridge TCR activation to downstream signaling mechanisms and formation of Nck-mediated signaling complex gives rise to the propagation of proximal TCR signals, leading to functional events.

However, conflicting data indicate that interaction between the CD3ε PRS and Nck is not required for T cell development and function (Szymczak et al., 2005). Mice lacking CD3ε PRS motif have normal number and percentage of T cell subsets in the thymus and spleen, with out apparent defect in positive or negative selection. Furthermore, the CD69 expression and proliferative response of mutant T cells to staphylococcal enterotoxin B (SEB) and anti-CD3 antibody is normal. Biochemical and structure studies have demonstrated that the Nck-CD3ε is capable of downregulating T cell activation by inhibiting of CD3ε ITAM phosphorylation and subsequent ITAM-dependent recruitment of downstream signaling molecules by Fyn and Lck kinases in vitro and/or reducing TCR cell surface expression upon physiological stimulation in mouse primary lymph node cells (Takeuchi, et al., 2008). In this context, Nck binds to a noncanonicalPxxDY region juxtaposed to the PRS in CD3ε chain. Accordingly, the PxxDy motif encompasses a putative internalization motif, YxxI/L, although this sequence may behave only as a weak internalization signal of TCR/CD3ε endocytosis (Borroto, et al., 1999). Moreover, it has been suggested that the

capacity of CD3ε to bind Nck may serve to amplify TCR signaling and to promote ITAM phosphorylation in response to weak TCR stimuli (Tailor, et al., 2008). In this scenario, strong MHC agonists triggered cytokine release in both naïve and differentiated murine CD8+ T cell types whereas weak agonists only affected differentiated cells. Proliferation of PRS-mutant naïve mouse T cells does not differ from wild-type CD3ε in response to strong agonists. The responsiveness may correlate with the ability of the agonist to elicit a CD3ε conformational change as measured by the ability of CD3ε to bind Nck. The mutation of the CD3εproline motif and the ITAM significantly impairs the response to weak antigens in differentiated but not naïve cells. Therefore, the importance of Nck-mediate T cell activation is still a matter of debate in terms of various stages of T cell development as well as types of antigenic stimuli.

Clinical implications

Previous studies have suggested the necessary role of Nck in the activation and function of human T cells. The information obtained help to understand, at least in part, the involvement of Nck in the control of T cells responses. The subsequent applications may contribute to finding a new approach for diagnosis or treatment in immunopathologic conditions. Recently, based on the blockage of TCR-Nck interaction, synthetic peptides that mimic the sequence of PRS have been demonstrated to block the Nck binding to CD3ε and the activation of T cells can then be inhibited (Alarcon, et al., 2012). Nck recruitment to the TCR is required to elicit a protective adaptive immune response to tumor antigens in vivo and to allow T cell proliferation in vitro and in vivo (Borroto, et al., 2014). Understanding the role of adapter molecule such as Nck would provide a novel approach to the development of specific immunosuppressive or immunomodulatory agents with fewer side effects for the treatment of diseases that are associated with the activation of T cells such as autoimmune diseases and diseases that are associated with rejection of allotransplants or xenotransplants of organs or tissues.

Conclusions

Nck is proposed to play an essential role in T cell activation. Two different models for the association of Nck with TCR/CD3 complex have been proposed. In an ITAM-requiring pathway, Nck is recruited to the membrane proximal site via interaction with SLP-76, forming the activation complex that is involved in actin cytoskeletal rearrangement and T cell activation. In a non-ITAM-requiring pathway, Nck directly binds to a PRS in CD3ε that exposes due to a conformational change in the TCR complex after TCR ligation. Nck recruits associated regulatory proteins to CD3ε and this event displays an alternative way for T cell activation. Therefore, Nck could be a candidate for development of immunosuppressive drugs for treatment of immunopathologic conditions in the future.

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