Intracellular Redistribution of Nucleolin upon Interaction with the CD3ε Chain of the T Cell Receptor Complex*

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T cell activation through the antigen receptor (TCR) involves the cytoplasmic tails of the CD3 subunits CD3γ, CD3δ, CD3ε, and CD3ζ. Whereas the biological significance of the cytoplasmic tails of these molecules is suggested, in part, by their evolutionarily conserved sequences, their interactions with signal transduction molecules are not completely understood. We used affinity chromatography columns of glutathione S-transferase fused to the CD3ε cytoplasmic tail to isolate proteins that specifically interact with this subunit. In this way, we identified the shuttling protein nucleolin as a specific CD3ε-interacting molecule. Using competition studies and affinity chromatography on peptide columns, we were able to identify a central proline-rich sequence as the nucleolin-interacting sequence in CD3ε. Transfection in COS cells of wild type CD3ε, but not of nonbinding mutants of CD3ε, resulted in redistribution of nucleolin from the nucleus and nucleoli to the cytoplasm. This property was transferred to a CD8 protein chimera by appending the cytoplasmic tail of CD3ε to CD8. We also found that nucleolin associated with the TCR complex. This association was increased upon TCR engagement, suggesting that the CD3ε/nucleolin interaction may have a role in T cell activation.

T cells respond to antigen via a polypeptide complex composed of ligand-binding T cell receptor (TCR) α and β chains (or γ and δ in γδ T cells) and the CD3 subunits CD3γ, CD3δ, CD3ε, and CD3ζ (1, 2). Unlike the TCR chains, the CD3 components have long cytoplasmic tails that associate with cytoplasmic signal transduction molecules. This association is mediated at least in part by a double tyrosine-based motif present in a single copy in the CD3γ, CD3δ, and CD3ε chains and in three copies in CD3ζ (3). This motif, named immune-receptor tyrosine-based activation motif (ITAM), becomes tyrosine phosphorylated during T cell activation by the Src family protein-tyrosine kinase Lck and/or Fyn (4–6). Tyrosine phosphorylated ITAM become docking sites for the Syk family protein-tyrosine kinase ZAP70 and other signal-transducing molecules. It is well established that antibody-mediated engagement of protein chimeras containing the cytoplasmic tail of either CD3ζ or CD3ε results in T cell activation (7–10). These data indicate that the cytoplasmic tail of one of these subunits can be sufficient to induce T cell activation. Regarding the role of CD3 subunits in T cell activation, most of the attention has been focused on the ITAM. However, the cytoplasmic tails of the CD3 subunits contain other evolutionarily conserved features that suggest ITAM-independent roles for them.

The CD3ε cytoplasmic tail, highly conserved (11, 12), can be tentatively subdivided into three regions; the N-terminal region contains a basic amino acid cluster, the central region contains a proline-rich sequence, and the C-terminal region contains the ITAM (13). The proline-rich sequence contains the SH3-binding consensus motif XPPXXP, and the C-terminal region contains the YXXLXXR endoplasmic reticulum (ER) retention sequence, which partially overlaps the ITAM (14, 15). Previous attempts to identify proteins that associate with the cytoplasmic tail of CD3ε have shown the specific interaction of a nuclear protein, topoisomerase IIβ, and a tyrosine-phosphorylated protein, CAST, with the N-terminal region of the CD3ε tail (13, 16).

Nucleolin is a major nuclear protein of exponentially growing eukaryotic cells that is directly involved in the regulation of ribosome biogenesis and maturation (17, 18). Nucleolin has a molecular mass of 100–110 kDa and is mainly found in the fibrillar components of the nucleoli where it associates with nascent preribosomal RNA. Numerous reports have implicated the involvement of nucleolin either directly or indirectly in the regulation of cell proliferation and growth, cytokinesis, replication, embryogenesis, and nucleogenesis (17, 18). Although predominantly localized in the nucleolus, nucleolin has also been found in the cytoplasm and at the plasma membrane, where it can function as a cell surface receptor for ligands as different as coxsackie B viruses and the complement inhibitor factor J (18–20). Because nucleolin acts as a shuttling protein between the cytoplasm and the nucleus, it might provide a mechanism for extracellular regulation of nuclear events.

Nucleolin activity is regulated by proteolysis, methylation, ADP-ribosylation, and phosphorylation by casein kinase II, Cdc2, PKC, cyclic AMP-dependent protein kinase, and ecto-protein kinase (17, 18). Nucleolin is cleaved by a leupeptin-sensitive protease that is tightly associated with it. It has been also suggested that nucleolin itself may possess a self-cleaving activity. In an attempt to identify novel CD3ε tail-interacting proteins, we have utilized affinity chromatography using glutathione S-transferase (GST) columns. In this way, we were able to characterize nucleolin as a major CD3ε-interacting protein that associates with the central proline-rich region. We also show...
herein that expression of CD3ε in a heterologous cell system results in loss of both nucleolin localization in the nucleolus and redistribution to the cytoplasm. A possible role of nucleolin/CD3ε interaction in TCR-mediated T cell activation is proposed.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—The COS-7 African green monkey cell line was grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum (Sigma). The human leukemic T cell line Jurkat was grown in RPMI 1640 medium supplemented with 5% fetal bovine serum.

The mouse monoclonal anti-human nucleolin antibody D3 (21) used in this study was a gift from Dr. B. Ballou (University of Pittsburgh, PA). The mouse monoclonal anti-CD8α B9.4 was donated by Dr. B. Malissen (Center d’Immunologie, Marseille-Luminy, France). The mouse monoclonal antibody SF34, specific for the extracellular domain of human CD3ε (22), was a gift from Dr. C. Terhorst (Beth Israel Deaconess Hospital, Boston, MA). The mouse monoclonal anti-human CD3 antibody UCHT1 was donated by Dr. P. Beverley (The Edward Jenner Institute for Vaccine Research, Berkshire, UK). Peptides 7 and 8, corresponding to amino acids 170–185 and amino acids 150–166 of human CD3ε respectively, were synthesized by the N-(9-fluorenylmethoxycarbonyl) (Fmoc) method and purified by HPLC.

**DNA Constructs**—To generate the GSTε fusion protein, a 165-base pair cDNA fragment, corresponding to the whole cytoplasmic tail of human CD3ε (amino acids 131–185), was generated by polymerase chain reaction. This fragment was digested and inserted into the XhoI and NotI sites of the plasmid pGEX-4T3 (Amersham Pharmacia Biotech). The truncated CD8α construct was prepared by polymerase chain reaction by introducing a stop codon after the second cytoplasmic amino acid of human CD8α. The polymerase chain reaction product was cloned into the XhoI and BamHI sites of the pSRα expression vector. The CD3ε construct expressing the extracellular and transmembrane domains of human CD8α fused to the cytoplasmic tail of CD3ε has been previously described (23) and was a gift from Dr. C. Terhorst.

**Affinity Chromatography**—To characterize proteins that interact with the cytoplasmic tail of CD3ε, a GSTε column was generated by absorbing 20 ml of GSTε-producing Escherichia coli lysate (resulting from a 1-liter culture) to 1 ml of glutathione-Sepharose (Amersham Pharmacia Biotech). A similar preabsorb column was prepared from GST-producing E. coli. A total of 4 × 10^9 Jurkat cells were lysed in 1% Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl, pH 7.8, 10 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin). A postnuclear supernatant of the Jurkat cell lysate was serially passed first through the GST and then through the GSTε columns. Bound proteins from [35S]methionine-labeled Jurkat cell lysates, we detected several major protein bands, including nucleolin and products of its partial proteolysis (Fig. 2A). Protein bands b and e yielded no sequence. To confirm that nucleolin associates to the cytoplasmic tail of CD3ε, the eluates from the GST and GSTε columns were immunoblotted with a specific monoclonal anti-nucleolin antibody. This antibody reacted with a 110-kDa protein band (protein a) present in the GSTε eluate but not in the GST eluate (Fig. 1B). These results showed that nucleolin and its partial proteolytic fragments specifically associate with the cytoplasmic tail of CD3ε in vitro.

**Nucleolin Binds to the Central Proline-rich Region of the Cytoplasmic Tail of CD3ε**—To map the binding site of nucleolin on the cytoplasmic tail of CD3ε, we used the monoclonal antibody APA1/1, which binds a well defined sequence within this tail. Using a GSTε column to pull down CD3ε-interacting proteins from [35S]methionine-labeled Jurkat cell lysates, we detected several major protein bands, including nucleolin and its partial degradation product of 68 kDa, and actin (Fig. 2A, mock). Incubation of the cell lysate with APA1/1 completely inhibited the binding to GSTε of the 110- and 68-kDa nucleolin forms but not the binding of actin or other contaminant proteins (Fig. 2A, APA1/1). A 75-kDa GSTε-associated protein was also completely displaced by APA1/1, but this protein probably represents a partial proteolysis product of nucleolin. This result indicated that the antibody APA1/1 specifically inhibits the association of nucleolin with the cytoplasmic tail of CD3ε.

In previous work (12), we mapped the binding site of APA1/1 to a 10-amino acid region in the central proline-rich region of the tail of CD3ε (Fig. 2B). This suggested that the binding site of nucleolin maps to the central region of the tail of CD3ε. To confirm this, a competition experiment was set up using a 17-mer synthetic peptide (peptide 8) that expands the APA1/1-binding site. Peptide 8 but not a control peptide of the same length expanding the C-terminal region of CD3ε (peptide 7) inhibited the binding of nucleolin to GSTε (Fig. 2A). Additional evidence that nucleolin binds the central proline-rich region of the tail of CD3ε was the finding that nucleolin binds to a column of immobilized peptide 8 but not of peptide 7 (Fig. 2C).

**Expression of CD3ε in an Heterologous Cell System Promotes Intracellular Redistribution of Nucleolin**—To determine whether the observed interaction of nucleolin with the cytoplasmic tail of CD3ε resulted in a change in the intracellular...
distribution of these proteins, CD3ε was transfected into COS cells, and the localization of transfected CD3ε and endogenous nucleolin was assessed by two-color immunofluorescence. Nucleolin was found in the nucleus and the nucleolus (Fig. 3A, red staining), whereas CD3ε was detected in the cytoplasm and nuclear membrane in an ER-characteristic pattern (Fig. 3A, green staining). Interestingly, both stainings were mutually exclusive, i.e. nucleolin staining was not observed in CD3ε-expressing cells. To determine whether the effect of CD3ε expression on nucleolin distribution correlated with its capacity to interact with nucleolin, different CD3ε mutants were assayed. Unlike wild type CD3ε, transfection with a truncated (tail-less) CD3ε construct did not alter nucleolin distribution (Fig. 3B). This inferred that expression of the cytoplasmic tail of CD3ε was necessary for nucleolin redistribution and suggested that the effect of CD3ε on nucleolin is mediated by its ability to interact with it. However, because the deletion of CD3ε tail resulted in a loss of its ER retention (Fig. 3B), it is also possible that the effect on nucleolin requires the localization of CD3ε in the ER rather than direct binding to nucleolin. To discriminate between these possibilities, mutant 9, a deletion mutant that lacks 10 amino acids of the central, proline-rich region of CD3ε (Fig. 2B) was assayed. This mutant has lost the capacity to interact with the antibody APA1/1 (12). Like wild type CD3ε, mutant 9 was also located in the ER (Fig. 3B). However, mutant 9 did not alter the nucleolar location of nucleolin, strongly suggesting that redistribution of nucleolin requires direct binding to CD3ε. Indeed, in cells that overexpress CD3ε, nucleolin was found to colocalize with CD3ε in the ER (Fig. 3B). These results indicate that CD3ε requires nucleolin binding capacity for its effect on nucleolin distribution. Nevertheless, localization of CD3ε to the ER seems to be required as well, because transfection of two C-terminal deletion mutants of CD3ε that result in loss of ER retention (14) did not cause CD3ε redistribution (data not shown).

Expression of a Protein Chimera Containing the Cytoplasmic Tail of CD3ε Results in Redistribution of Nucleolin—To determine whether expression of the cytoplasmic tail of CD3ε is sufficient to enable nucleolin relocalization, a protein chimera consisting of the cytoplasmic tail of CD3ε appended to the transmembrane and extracellular domains of CD8α (CD8αt) was obtained (23). As a control, a truncated mutant of CD8α lacking the cytoplasmic tail was used (CD8at). Although the CD8αt construct is in part retained in the ER because the cytoplasmic tail of CD3ε contains an ER retention sequence (14, 15), both CD8αt and CD8at were expressed on the cell surface. This allowed separation of transfected COS cells from untransfected cells by immunoselection with antibody-coated magnetic beads. As anticipated, the expression of the CD8αt chimera in the magnetic bead-selected COS cell population (65% CD8αt) led to the localization of nucleolin in the cytoplasm (Fig. 4). In contrast, in the nonselected population (95% CD8at) nucleolin was located in the nucleus and nucleolus. Expression of CD8at did not alter the nuclear localization of nucleolin (Fig. 4). This indicated that the cytoplasmic tail of CD3ε was sufficient to promote the intracellular redistribution of nucleolin.

Antibody-mediated TCR Cross-linking Increases Nucleolin Recruitment to the TCR—To determine whether the CD3ε/nucleolin interaction takes place in T cells and whether the interaction changes upon TCR engagement, the human T cell line Jurkat was stimulated with the anti-CD3 antibody UCHT1 followed by cross-linking with a secondary antibody. Mock-stimulated and stimulated cells were lysed, and immunopre-
Precipitation was carried out with the anti-CD3ε antibody SP34. Immunoblotting of the SP34 immunoprecipitates with anti-nucleolin antibody showed that nucleolin is associated to the TCR complex in nonstimulated T cells (Fig. 5). The association was increased in Jurkat cells stimulated with anti-CD3 antibody. These results show that the TCR complex, probably via CD3ε, interacts with nucleolin in T cells and that more nucleolin is recruited to the TCR complex when this is cross-linked

**DISCUSSION**

These results show that the cytoplasmic tail of human CD3ε interacts with nucleolin in vitro. We mapped the site of interaction to a central 10–17-amino acid proline-rich sequence within the cytoplasmic tail. Recently, Saito’s group (13, 16) has described the interaction of the cytoplasmic tail of CD3ε with two other proteins, topoisomerase IIβ and CAST, a tyrosine phosphorylated protein. Both proteins interact with the N-terminal part of the cytoplasmic tail of CD3ε, a region rich in basic amino acids. Therefore, the cytoplasmic tail of CD3ε appears to interact with different proteins along its sequence: with topoisomerase IIβ and CAST in the N-terminal region, with antibodies, suggesting that the CD3ε/nucleolin interaction may have a role in T cell activation.
with nucleolin in the central portion, and via the ITAM (C terminus) with ZAP70 and probably other SH2-containing proteins (23, 24).

Although at first glance it would seem unlikely that the cytoplasmic tail of CD3ε interacts with two nuclear proteins, topoisomerase IIβ and nucleolin, the interaction with both proteins might be facilitated by a possible location of CD3ε in the inner nuclear membrane (13). CD3ε contains a sequence at the N-terminal portion of the cytoplasmic tail reminiscent of a nuclear localization signal. Moreover, CD3ε has a double arginine sequence in the central portion of its cytoplasmic tail that is reminiscent of the signal sequence responsible for the localization of glycoprotein B of human cytomegalovirus (a transmembrane protein) in the inner nuclear membrane (25). Indeed, CD3ε has been located in the nucleus (13), although the role of the nuclear localization signal and the presence of a nuclear inner membrane localization signal have not yet been demonstrated. Therefore, the intracellular location site of CD3ε for its interaction with nucleolin, and topoisomerase IIβ, could conceivably be the nucleus.

A second possible location for the interaction of CD3ε with nucleolin could be the cytoplasm. Nucleolin has been shown to shuttle between the cytoplasm and the nucleus. Indeed, the interaction of nucleolin with several cytoplasmic proteins and even plasma membrane proteins has been reported (18–20). We have shown in this study that nucleolin interacts with the TCR complex in T cells, probably through CD3ε. Although we cannot discriminate whether nucleolin interacts with the TCR at the plasma membrane or with the intracellular pool of TCR, the fact that antibody engagement of the TCR results in increased recruitment of nucleolin suggests that nucleolin interacts with the TCR at the plasma membrane. In addition, our present results reveal that expression of CD3ε in COS cells results in the loss of nuclear localization of nucleolin and, in some cases, in relocalization to the cytoplasm. This effect is dependent on the expression of the nucleolin-interacting sequence in the central portion of CD3ε and can be transferred by appending the CD3ε tail to the extracellular and transmembrane domains of an irrelevant protein. Thus, the correlation between nucleolin-binding capacity and nucleolin redistribution induced by CD3ε indicates that CD3ε promotes nucleolin relocalization by directly binding nucleolin.

Similar to the effect of CD3ε expression, it has been described that infection by poliovirus causes a relocalization of nucleolin to the cytoplasm, perhaps by binding of nucleolin to the 3′ noncoding region of poliovirus RNA (26). However, although the role of nucleolin binding to poliovirus RNA seems to be that of promoting assembly of new virions, the role of CD3ε-induced relocalization of nucleolin is not well understood yet. For the topoisomerase IIβ-CD3ε interaction, it has been proposed that it could be involved in signal transduction because topoisomerase II inhibitors up-regulate IL-2 production and apoptosis (13). Therefore, by binding topoisomerase IIβ, CD3ε could participate in TCR-induced growth arrest and apoptosis of T cells. It has also been described that nucleolin binds specifically to a Jun N-terminal kinase response element and that this binding is required for interleukin-2 mRNA stabilization induced by T cell activation signals (27). Our observation that the association of nucleolin to the TCR is increased upon antibody-mediated cross-linking of the TCR suggests that nucleolin/CD3ε interaction may have a role in T cell activation. Although a positive effect on T cell activation cannot be excluded, we favor the hypothesis that recruitment of nucleolin to the TCR through CD3ε and redistribution of nucleolin to the cytoplasm may have roles in TCR-induced growth arrest, given the important roles for cell survival and proliferation that nucleolin plays at the nucleus (17, 18).

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Fig. 4. Effect of CD3ε tail expression on nucleolin levels and localization. Expression of a CD8ε chimera but not of truncated CD8 resulted in redistribution of nucleolin to the cytoplasm. COS cells transfected with the CD8ε chimera were immunoselected. The selected and nonselected populations were stained with anti-CD8 and anti-nucleolin antibodies and examined under fluorescence microscopy. Notice that in the selected CD8ε-expressing COS cells nucleolin is distributed to the cytoplasm, whereas in the nonselected population, nucleolin is located in the nucleus. In COS cells expressing truncated CD8 nucleolin remained in the nucleus.

Fig. 5. TCR engagement increases nucleolin association to the TCR complex. Jurkat cells were stimulated with a combination of the anti-CD3 antibody UCHT1 and a cross-linking second antibody for 5 min (+ stimulus) or left untreated (− stimulus). The cells were then lysed and immunoprecipitation was carried out with anti-CD3ε antibody SP35. Immunoprecipitates were resolved by SDS-PAGE and immunoblotting was performed with the anti-nucleolin antibody. A sample of the total lysate was run in parallel as a control. NIS precipitation with nonimmune serum. H, immunoglobulin heavy chain.
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