Expression of the human immunodeficiency virus (HIV) Nef protein has been linked to both decreased cell surface expression of CD4 and an impairment of signal transduction. The recently reported association of Nef with an unidentified serine kinase provides a clue as to how Nef might exert its effects. Considering the key role of protein kinase C (PKC) in T cell activation, we investigated the possibility that Nef interacts with PKC. Our results, using two approaches for detecting interactions between Nef and PKC isozymes in Jurkat cells, show that Nef interacts preferentially with εPKC. The interaction of Nef and εPKC is independent of calcium, enhanced by phospholipid activators of PKC and not affected by a PKC pseudosubstrate peptide. Phorbol 12-myristate 13-acetate and phytomagensulbin stimulation of Jurkat cells expressing Nef fails to produce the usual translocation of εPKC from the cytosol to the particulate fraction; translocation of βPKC and εPKC was unaffected. Indeed, there appears to be a net loss of εPKC in Nef-expressing cells following stimulation. The loss of εPKC, which may be a result of inhibition of its binding to RACKs due to Nef binding, could contribute to the various impairments of T cell function associated with HIV infection and Nef expression.

Nef is one of seven accessory proteins encoded by the human and simian immunoindemency viruses (HIV and SIV). The 25-kDa nonmyristylated and the 27-kDa myristylated forms occur in the cytoplasm, the nucleus, and the plasma, Golgi, and perinuclear membranes (1–7). The effects of Nef on virus replication, latency, and host cell functions and survival are unclear (8). Nef has been reported to down-regulate the level of CD4 on the surface of infected T cells (9, 10) and to block T cell signaling pathway. However, the intracellular substrates and RACKs that εPKC interacts with have yet to be determined. In this work, we have explored the possibility that Nef interacts with PKC and more specifically with εPKC. Our results suggest that Nef may interfere with the interaction of εPKC with its endogenous anchoring proteins and that this inhibition may result in the net loss of the isozyme.
Pseudosubstrate inhibitor peptide is a highly specific inhibitor of PKC that is not isozyme-specific. The results are expressed as the averages of duplicate assays and are from one of three experiments with similar results.

Polyclonal antibodies were diluted 1:300. Previous experiments found that these were the most abundant PKC isozymes in these cells. The Western blots were developed using chemiluminescence (ECL; Amer sham Corp.)

Co-immunoprecipitations from Jurkat Cells—Co-immunoprecipitations were performed on the 0.1% Triton X-100 extracts of approximately 5 x 10^7 Jurkat cells that had or had not been exposed to PMA and PHA for 5 min. Cells were stimulated with 100 ng/ml PMA (Sigma) and 2 μg/ml PHA (Sigma). The Nef-expressing cell line (133) and the J25 parent cell line were used for the immunoprecipitations. Construction of cell lines and their culture are described in Luria et al. (11). Following stimulation, the cell extracts were prepared as above, except for the addition of 0.1% Triton. 6–8 μl of antibodies to Nef (11), αPKC (a generous gift from Isakov and Altman (19)) or rabbit preimmune serum were incubated with the extracts for 2 h. The antibody-antigen complex was then precipitated with protein A-Sepharose (Pharmacia). Alternatively, the antibodies were biotinylated with SS-NHS-Biotin (Pierce). The antibody-antigen-biotin complex was then precipitated with protein A-Sepharose (Pharmacia). The antigen complex was then precipitated with avidin coupled to agarose (Pierce). The antigen complex was then precipitated with avidin coupled to agarose (Pierce). The antigen complex was then precipitated with avidin coupled to agarose (Pierce). The antigen complex was then precipitated with avidin coupled to agarose (Pierce).

PKC Translocation Assays—To determine the translocation of PKC isozymes (30, 31), equal amounts of protein (as determined by Bradford assays; Bio-Rad) from the cytosolic and Triton soluble particulate fractions of unstimulated and stimulated cells were loaded onto 10% SDS-polyacrylamide gels for Western analysis for β, ε, and γPKC isozymes. The cellular fractions were produced from cell homogenates by a 30-min 100,000 × g centrifugation followed by a 0.1% Triton extraction of the pellet and recentrifugation. The supernatant from the final centrifugation was used as the Triton soluble particulate fraction. The larger molecular weight protein detected by the antibody to γPKC, which translocates with stimulation, may be α or βPKC as reported by others (32). The Triton soluble particulate fraction does not contain the nuclei or other cellular components not extracted with 0.1% Triton. Western analysis of the Triton nonextracted pellet from unstimulated or stimulated cells revealed little iPKC remaining in the pellet (results not shown).

RESULTS AND DISCUSSION

To test for an interaction between Nef and PKC, Nef was expressed as a GST fusion protein in E. coli, purified by adsorption on glutathione-agarose beads, and incubated with the cytosolic fraction of Jurkat cells. PKC associated with the GST-Nef fusion protein was assayed by measuring the phosphorylation of a specific PKC substrate peptide (Fig. 1). PKC activity was readily detected in the proteins extracted with GST-Nef but not with GST. Phosphorylation occurred only in the presence of PKC activators and was inhibited by a specific PKC inhibitor, the PKC pseudosubstrate peptide (28). Maximal phosphorylation activity occurred in the presence of phosphatidylserine, diacylglycerol, and EGTA, suggesting that a calcium-independent PKC isozyme binds Nef. A decrease in kinase activity in the presence of calcium was not observed in all experiments and may be the result of increased degradation of the enzyme due to the calcium in some of the experiments.

The PKC family consists of at least eleven isozymes (33), five of which (α, β, ε, η, and ζ) are clearly detected by Western blot analysis in Jurkat cells (Fig. 2A, cytosol). To identify which PKC isozyme bound to Nef, the purified GST-Nef and associated proteins recovered after incubation with Jurkat cell cytosol were electrophoresed, and the protein bands were analyzed with antisera specific for the five PKC isozymes (Fig. 2A). Of the five PKC isozymes detected in Jurkat cells, only iPKC bound to the GST-Nef fusion protein; lower amounts of the PKC isozymes bound to GST alone due to nonspecific interactions. Further evidence supporting our conclusion that iPKC binds Nef is the observation that the addition of increasing amounts of purified, unbound Nef proportionally lowered the binding of iPKC to the fusion protein (Fig. 2B). In addition, treatment of the GST-Nef fusion protein with thrombin, which cleaves Nef from GST, eliminated the binding of iPKC to the glutathione beads (results not shown).

We have noted that under our conditions only a fraction of
the cytosolic δPKC (about 5%) is bound to the GST-Nef, even though the amount of GST-Nef is in molar excess over the amount of δPKC. In addition, PKC activators phosphatidylserine and diacylglycerol were present in sufficient concentrations to fully activate the PKC. Moreover, when the cytosolic fraction, which had already been reacted with GST-Nef, was reincubated with fresh GST-Nef, there was no additional binding of δPKC. This suggests that δPKC in the cytosolic fraction is heterogeneous with respect to its ability to bind Nef.

Co-incubation of PKC with phosphatidylserine, diacylglycerol, and some isozymes with calcium as well results in a conformational change in the protein that allows for substrate phosphorylation and binding to RACKs or other PKC binding proteins (23, 28). Therefore, we sought to determine if δPKC needs to be activated to bind Nef. To this end GST-Nef, immobilized on glutathione beads, was incubated with Jurkat cell cytosol in the presence of phosphatidylserine and diacylglycerol (PS/DAG), calcium (Ca), EGTA, or the PKC pseudosubstrate peptide. δPKC binding was determined as above (approximate molecular mass of detected protein is 80 kDa).

![Diagram of binding conditions](image)

**Fig. 3.** δPKC binding to the GST-Nef fusion protein requires phospholipids and is not inhibited by a PKC pseudosubstrate peptide. GST or GST-Nef fusion proteins immobilized onto glutathione beads were incubated with Jurkat cell cytosol in the presence of phosphatidylserine and diacylglycerol (PS/DAG), calcium (Ca), EGTA, or the PKC pseudosubstrate peptide. δPKC binding was determined as above (approximate molecular mass of detected protein is 80 kDa).

In vivo activation of PKC generally results in the translocation of the isozymes from the cytosol to the particulate fraction with no net change in the total amount of the enzymes (30, 31). However, in Jurkat cells expressing Nef, the total amount of δPKC declined after 5 min of PMA and PHA stimulation (Fig. 5A); by comparison the total level of βPKC remained unchanged after stimulation. To examine this isozyme-specific difference further, the translocation of four PKC isozymes was compared in PMA- and PHA-activated control and Nef-expressing Jurkat cells (Fig. 5B). Translocation of β, ε, and δPKC isozymes was observed after 5 min of PMA and PHA stimulation.
in the level of possibly, the loss of u isozymes was virtually unchanged in the Jurkat cells expressing Nef. The loss of PKC declined in Nef-expressing cells following PMA and PHA stimulation, whereas total βγPKC levels remain unchanged (A; approximate molecular masses: γPKC, 80 kDa; βγPKC, 82 kDa; Nef, 27 kDa). Triton extracts of total cell homogenates were prepared of the stimulated and unstimulated Nef-expressing cells and subjected to Western analysis. The levels of Nef in the two cell extracts were also determined to demonstrate that equal amounts of protein were loaded in both lanes. Control Jurkat cells or Nef-expressing cells were stimulated with PMA and PHA and fractionated into cytosol and Triton-soluble particulate fractions to further analyze the loss of PKC. The total level of γPKC before and after stimulation in the two cellular fractions (approximate molecular masses: γPKC, 80 kDa; βγPKC, 82 kDa; εPKC, 90 kDa; γPKC, 69 kDa, lower band) are the results from one of six independent experiments with similar results.

The loss of γPKC following PMA and PHA stimulation of Nef-expressing cells may be a result of the isozyme's interaction with Nef. This could result from the failure of γPKC to bind to its endogenous anchoring protein(s) or RACKs (21–23) following activation of the PKC. Activated PKC has been shown to be more sensitive to proteolysis (36). Inhibition of binding of activated PKC to its endogenous binding proteins or RACKs may leave the PKC more susceptible to degradation. Indeed, an inhibition of translocation and a net loss of PKC following activation was observed in oocytes in which translocation inhibitors (either a purified RACK protein or peptides based upon sites of interaction between PKC and RACKs) were introduced by microinjection (35, 37, 38). Nef may act as an inhibitor of γPKC translocation in lymphocytes in a similar manner. Quite possibly, the loss of γPKC or its inappropriate binding to its targets could account for the various phenotypic impairments of T cell function associated with Nef and HIV infection. Further work is required to determine the molecular basis for the loss of γPKC in cells expressing Nef and how this disruption in signal transduction affects T cell activation.

Fig. 5. Nef expression in Jurkat cells correlates with the specific loss of γPKC following PMA and PHA stimulation. The total level of γPKC declined in Nef-expressing cells following PMA and PHA stimulation, whereas total βγPKC levels remain unchanged (A; approximate molecular masses: γPKC, 80 kDa; βγPKC, 82 kDa; Nef, 27 kDa). Triton extracts of total cell homogenates were prepared of the stimulated and unstimulated Nef-expressing cells and subjected to Western analysis. The levels of Nef in the two cell extracts were also determined to demonstrate that equal amounts of protein were loaded in both lanes. Control Jurkat cells or Nef-expressing cells were stimulated with PMA and PHA and fractionated into cytosol and Triton-soluble particulate fractions to further analyze the loss of γPKC. Samples containing equal amounts of protein were analyzed by Western blot to determine the relative levels of γ, β, ε, and γPKC before and after stimulation in the two cellular fractions (approximate molecular masses: γPKC, 80 kDa; βγPKC, 82 kDa; εPKC, 90 kDa; γPKC, 69 kDa, lower band). The results are from one of six independent experiments with similar results.

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