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Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes

(acquired immunodeficiency syndrome/phosphorylation)

EARL T. SAWAI*, ANDREAS BAUR†, HEIDI STRUBLE*, B. MATIJA PETERLIN†, JAY A. LEVY*, AND CECILIA CHENG-MAYER*

*Cancer Research Institute, Department of Medicine, and †Howard Hughes Medical Institute, University of California, San Francisco, CA 94143-0128

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ABSTRACT **With T-cell lines constitutively expressing Nef from the SF2 strain of human immunodeficiency virus type 1 (HIV-1_{SF2}) in the form of a hybrid CD8–Nef fusion protein or T-cell lines chronically infected with HIV-1_{SF2}, a cellular serine kinase was found that specifically associates with Nef. Proteins of 62 kDa and 72 kDa, which coimmunoprecipitated with Nef, were phosphorylated in *in vitro* kinase assays. This Nef-associated serine kinase activity was not blocked by inhibitors of protein kinase C or protein kinase A and was lost when Nef was truncated at amino acid 94 or 99. These findings present evidence that a serine kinase activity is associated with Nef expressed in human T lymphocytes.**

Nef was first identified as an open reading frame that overlaps with the 3′ long terminal repeat in the human immunodeficiency virus (HIV) type 1 (HIV-1) (1). This gene is conserved in HIV-2 and in the simian immunodeficiency virus (2, 3). The name *nef* (negative factor) was derived from initial reports that its expression in T- and monocytic cell lines was associated with reduced levels of viral replication (4–7). Subsequent studies demonstrated that Nef acts as a transcriptional silencer (8–11) and inhibits mitogenic induction of transcription factors NF-κB and AP-1 (12, 13). The function of the Nef protein in suppressing HIV replication and expression from the long terminal repeat, however, is controversial (14, 15), and increased levels of viral replication in peripheral blood lymphocytes have been observed in the presence of Nef (16, 17). In simian immunodeficiency virus-infected rhesus monkeys, moreover, expression of Nef is required for maintenance of high viral load and progression to AIDS (18). Thus, the true function of Nef is still not known.

The *nef* gene product is a small (27 kDa) myristoylated phosphoprotein found in the cytoplasm and inner plasma membrane (1, 19, 20). Myristoylation has been reported to be important for its functional activity (11, 17). Earlier biochemical studies indicated that Nef resembles Ras, having both GTP-binding and GTPase activities (20, 21). These findings, however, have not been confirmed by others (22–25). In addition, Nef has been noted to undergo autophosphorylation in the presence of GTP and ATP (20, 21).

Several reports indicate that Nef down-regulates both CD4 and interleukin 2 expression in some T-cell lines (20, 26–30). Moreover, Nef has been observed to inhibit thymocyte maturation in transgenic mice (31). Recently, it was observed that expression of a hybrid CD8–Nef (SF2) fusion protein on the inner surface of the plasma membrane of T cells resulted in an activated phenotype (A.B., E.T.S., P. Dazin, W. J. Fantl, C.C.-M., and B.M.P., unpublished work). The activation markers CD69 and CD25 were found to be induced in these cells.

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The elucidation of Nef function requires the identification and characterization of its potential intracellular targets. To facilitate these studies, T-cell lines that constitutively express the hybrid CD8–Nef (SF2) protein were used in biochemical analyses. Our studies show that a cellular serine kinase activity specifically coimmunoprecipitates with Nef.

MATERIALS AND METHODS

Cells and Antibodies. Jurkat cell lines that constitutively express the hybrid CD8–Nef (SF2) protein (A.B. *et al.*, unpublished work) and a hybrid CD8–antisense Nef protein were cultured in RPMI 1640 medium/10% fetal bovine serum/1% glutamine/Geneticin at 250 μg/ml (G418; GIBCO/BRL). HUT 78 cells chronically infected with HIV-1_{SF2} (E-line) (32) were grown in the same medium without G418. Normal rabbit serum (NRS) was obtained from ICN. The anti-CD8 monoclonal antibody Leu2a was obtained from Becton Dickinson. Rabbit anti-SF2 Nef serum was provided by Chiron.

Generation of Hybrid CD8–Nef-Expressing Cell Lines. To establish a system in which the expression of Nef could be easily monitored, T-cell lines that constitutively express HIV-1_{SF2} Nef as a chimeric fusion protein were generated (A.B. *et al.*, unpublished work). CD8–Nef chimeras were constructed by fusing an intact *nef* gene to the extracellular and transmembrane domains of CD8 (Fig. 1*A*). As a control, a plasmid containing the extracellular and transmembrane domains of CD8 fused to the *nef* gene in the antisense orientation was also made (Fig. 1*A*). These constructions were transfected into the human T-cell line Jurkat, and stable transformants that express the two forms of hybrid Nef proteins were selected by resistance to G418. The expression of hybrid CD8–Nef on the plasma membrane was monitored by flow cytometry.

Metabolic Labeling, Immunoprecipitation, and SDS/PAGE. Metabolic labeling with [^{35}S]methionine and [^{35}S]cysteine and immunoprecipitation analyses and SDS/PAGE were done as described (33), except that kinase extraction buffer (KEB) containing 0.5% (vol/vol) Nonidet P-40, 2 mM EDTA, 137 mM NaCl, 50 mM Tris·HCl (pH 8.0), 10% (vol/vol) glycerol, 2 mM sodium orthovanadate, 100 μM leupeptin (Boehringer Mannheim), and aprotinin at 2 μg/ml (Boehringer Mannheim) was used to extract cells and wash the immunoprecipitates.

***In Vitro* Kinase Assays, Phosphoamino Acid Analyses, and Partial V8-Protease Mapping of Phosphopeptides.** Immunoprecipitates of unlabeled cellular extracts were resuspended in kinase assay buffer [50 mM Tris·HCl (pH 8.0)/100 mM NaCl/1% (vol/vol) Triton X-100/5 mM $MgCl_2$] containing [γ-^{32}P]ATP at 200 μCi/ml (ICN) (1 Ci = 37 GBq) and incubated for 5 min at room temperature. The immunopre-

Abbreviations: HIV, human immunodeficiency virus; PKC, protein kinase C; PKA, protein kinase A; NRS, normal rabbit serum.

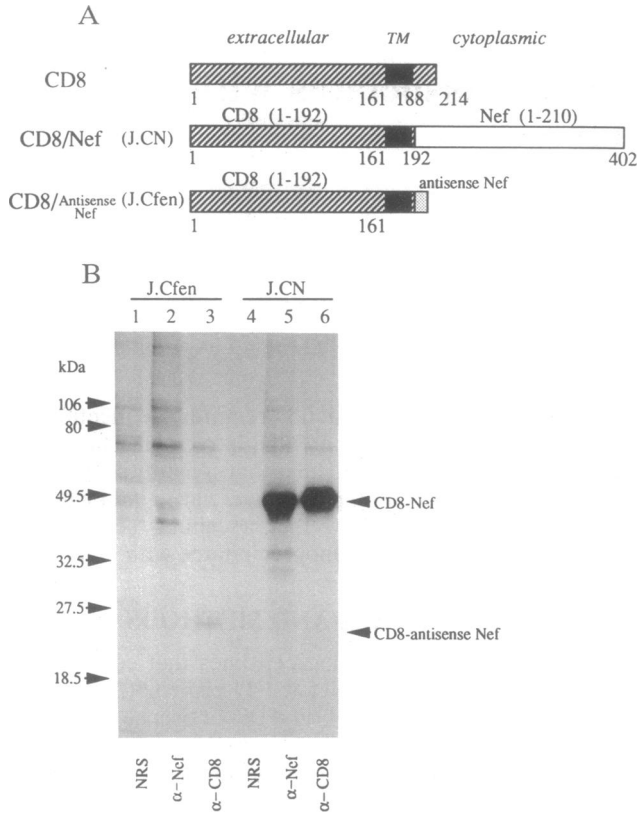

FIG. 1. Expression of hybrid CD8–Nef proteins in Jurkat cells. (*A*) Diagram of hybrid CD8–Nef and CD8–antisense Nef proteins. The CD8 protein is 214 amino acids in length. The N-terminal 192 amino acids of CD8, which contain the entire extracellular and transmembrane (TM) domain, were fused to Nef of HIV-1_{SF2} from amino acids 1 to 210. As a control, the N-terminal 192 amino acids of CD8 were fused to the antisense orientation of the *nef* gene. Jurkat cell lines that express each of the constructs are indicated in parentheses. (*B*) Jurkat cells expressing the hybrid CD8–antisense SF2 Nef protein (lanes 1–3) and the hybrid CD8–Nef protein (lanes 4–6) were metabolically labeled with ^{35}S, as described. Cells were extracted, and immunoprecipitation analyses were done by using NRS (lanes 1 and 4), polyclonal anti-SF2 Nef rabbit serum (α-Nef, lanes 2 and 5), or an anti-CD8 monoclonal antibody, Leu2a, (α-CD8, lanes 3 and 6). Positions of the hybrid CD8–Nef protein and the hybrid CD8–antisense Nef protein are indicated at right, and positions of molecular mass standards are indicated at left.

cipitates were washed with KEB buffer, and the proteins were analyzed by SDS/PAGE. Protein kinase C (PKC) activity was assayed by using a protein kinase assay system (BRL) as described by the manufacturer. The protein kinase inhibitors H7 (Sigma), H89 (CalBiochem), and PKC pseudosubstrate peptide (BRL) were used in the *in vitro* kinase assay.

To determine the identity of the phosphorylated residues, phosphoamino acid analyses were done on each of the phosphorylated substrates from the *in vitro* kinase assay, as described (34). Proteins phosphorylated in the *in vitro* kinase assay were analyzed by partial V8-protease mapping as described (35).

RESULTS

HIV-1_{SF2} Nef Is Expressed as a Chimeric Fusion Protein in Jurkat Cells. Clonal populations of Jurkat cells expressing CD8–antisense Nef (line J.Cfen) and CD8–Nef (line J.CN) were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine. Immunoprecipitations with either Nef-specific polyclonal rabbit serum or anti-CD8 monoclonal antibodies revealed a specific 49-kDa band, consistent with the predicted size for the hybrid CD8–Nef protein, in J.CN cells (Fig. 1*B*). Pulse-chase analyses that were done to assess the stability of the fusion protein showed that the metabolic half-life of the hybrid CD8–Nef protein (8 hr) was comparable to that of the native Nef expressed in HUT 78 cells chronically infected with HIV-1_{SF2} (E-line; data not shown).

A Cellular Kinase Activity Is Associated with the Hybrid CD8–Nef. Because previous studies with bacterial or *in vitro* synthesized Nef suggested that Nef may be associated with a kinase activity (20, 21, 36), *in vitro* kinase assays were done on immunoprecipitates containing the hybrid CD8–Nef protein. Two specific phosphorylated substrates of 62 kDa and 72 kDa were seen to coimmunoprecipitate with the hybrid CD8–Nef protein from J.CN extracts (Fig. 2*A*, lanes 11 and 12). Although the major phosphorylated protein appeared as a doublet of 62 kDa, the minor phosphorylated protein appeared as a triplet of 72 kDa. Neither of these phosphorylated substrates was observed with a control antibody (NRS, Fig. 2*A*, lane 10) or with immunoprecipitates from the control cell line J.Cfen (Fig. 2*A*, lanes 7–9). A weakly phosphorylated band similar in size to that of the hybrid CD8–Nef fusion protein was present in immunoprecipitates from J.CN cells (Fig. 2 *A*, lane 11, and *B*, lane 5). This band, however, was not observed consistently with anti-CD8 monoclonal antibodies (Leu2a) or with the polyclonal rabbit anti-Nef serum.

Characterization of the Protein Kinase Associated with Nef. Because Nef has been reported to autophosphorylate (20, 21), it is important to determine whether the 62-kDa phosphorylated protein represents a hyperphosphorylated form of the CD8–Nef molecule that exhibits slower mobility on SDS/PAGE gels. An *in vitro* kinase assay with unlabeled ATP was, therefore, performed on immunoprecipitates of ^{35}S-labeled CD8–Nef (Fig. 2*B*, lane 3). For comparison, a standard *in vitro* kinase assay was done in parallel (Fig. 2*B*, lanes 4 and 5). The addition of unlabeled ATP to the kinase assay did not affect the migration of the hybrid CD8–Nef protein on SDS/PAGE gels (compare lane 3 with lanes 2 and 5), indicating that the 62-kDa phosphorylated substrate is not a hyperphosphorylated form of the hybrid CD8–Nef protein. In addition, the 62-kDa protein is not the IgG heavy chain because it exhibited a slower mobility than IgG heavy chain on silver-stained gels (data not shown). These findings indicate that a kinase activity, which phosphorylates substrates of 62 kDa and 72 kDa, is present in immunoprecipitates of the hybrid CD8–Nef protein. However, Nef does not appear to autophosphorylate or to be phosphorylated in this assay.

To determine the specificity of the Nef-associated kinase, phosphoamino acid analyses were done on the 62-kDa and 72-kDa phosphoproteins immunoprecipitated from J.CN cells. Results showed that these proteins are phosphorylated solely on serine residues (Fig. 2 *C* and *D*, respectively). These findings indicate that a serine kinase coimmunoprecipitates with the hybrid CD8–Nef protein. Furthermore, this kinase utilizes [γ-^{32}P]ATP but does not utilize [γ-^{32}P]GTP (data not shown).

Because previous reports indicated that Nef is phosphorylated by PKC (20, 21), it was important to determine whether PKC was responsible for the Nef-associated kinase activity. The PKC inhibitor H7, a protein kinase A (PKA) inhibitor H89, and PKC pseudosubstrate peptides were preincubated with CD8–Nef immunoprecipitates before performing the *in vitro* kinase assay. Results from these experiments showed that the kinase activity is not due to PKC or PKA because no inhibition was seen with H7, H89, or the PKC pseudosubstrate peptide (Fig. 3). In contrast, the same concentrations of kinase inhibitors effectively inhibited the activity of purified PKC (data not shown).

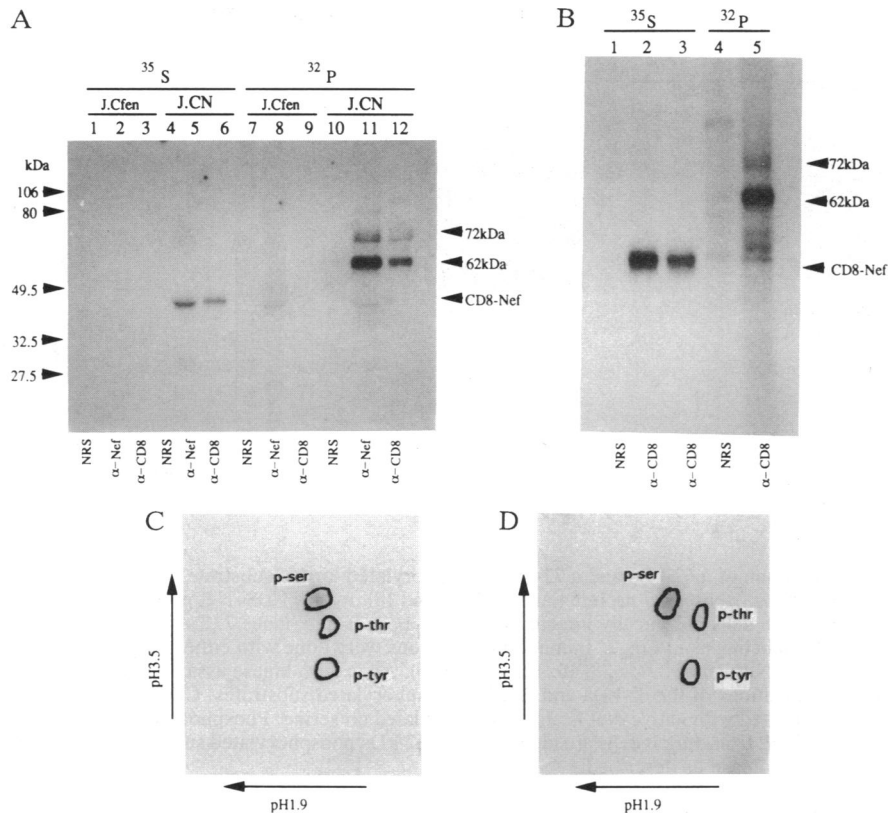

FIG. 2. Coimmunoprecipitation of a kinase activity from CD8–Nef immunoprecipitates. (*A*) Cells expressing the hybrid CD8–antisense Nef (lanes 1–3, 7–9) or the hybrid CD8–Nef protein (J.CN, lanes 4–6, 10–12) were extracted with (lanes 1–6) or without (lanes 7–12) metabolic labeling. Immunoprecipitations were done by using NRS (lanes 1, 4, 7, and 10), anti-Nef (lanes 2, 5, 8, and 11), or anti-CD8 (lanes 3, 6, 9, and 12). *In vitro* kinase assay was done on the unlabeled immunoprecipitates (lanes 7–12). The band corresponding to CD8–antisense Nef (lane 3) is faint on the photograph (but see Fig. 1*B*, lane 3, as a reference). (*B*) Immunoprecipitations were done on ^{35}S-labeled (lanes 1–3) or unlabeled (lanes 4 and 5) extracts using NRS (lanes 1 and 4) or an anti-CD8 monoclonal antibody, Leu 2a [α-CD8, lanes 2, 3, and 5). *In vitro* kinase assays were done on the immunoprecipitates from the unlabeled cell extracts (lanes 4 and 5), and in lane 3, 1.0 mM unlabeled ATP was added. Note that the mobility of the hybrid CD8–Nef protein does not change when incubated with unlabeled ATP. (*C* and *D*) The 62-kDa and 72-kDa proteins are phosphorylated on serine residues. Phosphoamino acid analyses were done on the 62-kDa (*C*) and 72-kDa (*D*) phosphoproteins that coimmunoprecipitate with CD8–Nef. Arrows indicate the direction of electrophoresis, and the pH of the buffer used for each dimension is indicated along each axis. Circles indicate positions of unlabeled phosphoserine (p-ser), phosphothreonine (p-thr), and phosphotyrosine (p-tyr) standards.

The Serine Kinase Activity Is Also Associated with Nef in Cells that Are Chronically Infected with HIV-1. To determine whether the kinase activity observed in the hybrid CD8–Nef immunoprecipitates was also associated with native Nef, *in vitro* kinase assays were performed on immunoprecipitates of Nef from chronically infected HUT 78 cells (E-line; Fig. 4*A*). As observed with the hybrid CD8–Nef-expressing J.CN cell line, phosphorylated proteins of 62 kDa and 72 kDa were also detected in the Nef immunoprecipitates from the E-line (Fig. 4*A*, lanes 12 and 16). Moreover, native Nef does not serve as a substrate for the serine kinase because no phosphorylated proteins were observed in the region of Nef on SDS/PAGE (E-line cells continually express two forms of Nef ranging from 25 to 27 kDa; Fig. 4*A*, compare lanes 8 and 16). These results confirm the above observation that Nef is not autophosphorylated in the *in vitro* kinase assay. In further support of these findings with native Nef, similar results were obtained using HUT 78 cells acutely infected with HIV-1_{SF33} (data not shown).

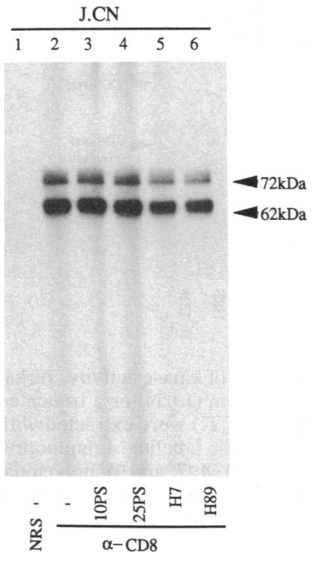

FIG. 3. Nef-associated kinase is not inhibited by PKC or PKA inhibitors. *In vitro* kinase assays were done on immunoprecipitates from J.CN cell extracts in the absence (−, lanes 1 and 2) or presence (lanes 3–5) of PKC inhibitors or a PKA inhibitor (lane 6). PKC pseudosubstrate peptide [10 PS (10 mM) and 25 PS (25 mM); lanes 3 and 4], 10 μg of H7 (lane 5), and 10 μg of H89 (lane 6) were used.

Phosphoamino acid analyses were done on the 62-kDa protein from immunoprecipitates containing native Nef (Fig. 4*B*). The data indicate that, like the 62-kDa protein that coimmunoprecipitates with the hybrid CD8–Nef protein from Jurkat cells, the 62-kDa protein in Nef immunoprecipitates from E-line is phosphorylated exclusively on serine residues. Therefore, native Nef from infected T cells and the hybrid CD8–Nef proteins both interact with a serine kinase that phosphorylates a 62-kDa substrate.

Because Nef from both infected- (E-line) and CD8–Nef-expressing (J.CN) T lymphocytes associates with a phosphorylated 62-kDa protein, partial V8-protease maps of each

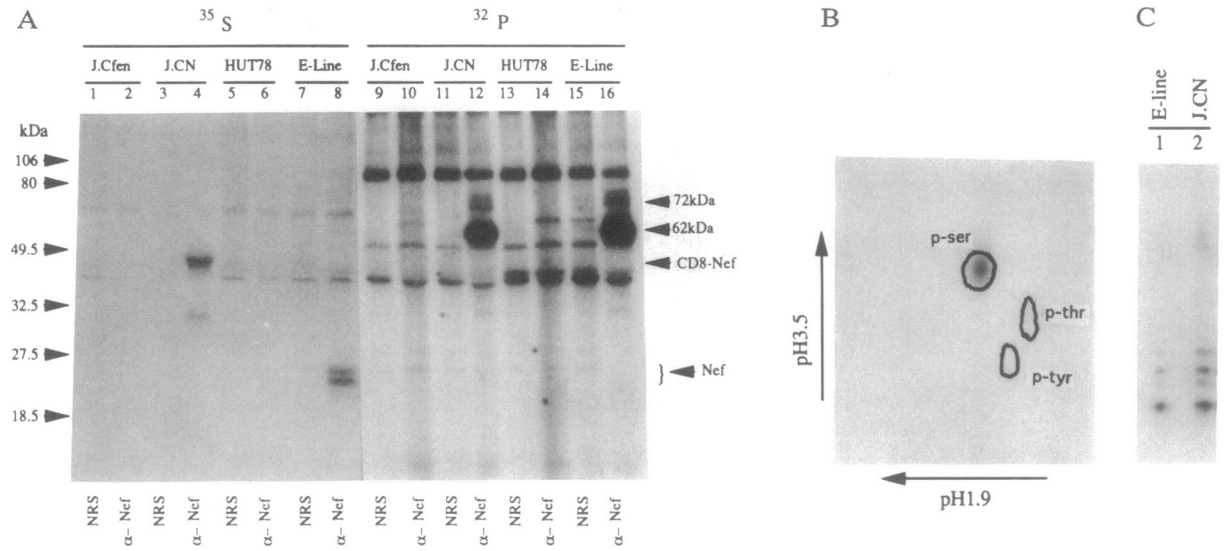

FIG. 4. (*A*) Coimmunoprecipitation of a 62-kDa and a 72-kDa phosphorylated kinase substrate from immunoprecipitates of HIV-1_{SF2} Nef. Jurkat cells expressing hybrid CD8–antisense Nef protein (lanes 1, 2, 9, and 10), hybrid CD8–Nef protein (lanes 3, 4, 11, and 12), HUT 78 cells (lanes 5, 6, 13, and 14), and HUT 78 cells chronically infected with HIV-1_{SF2}, E-line (lanes 7, 8, 15, and 16) were extracted with (lanes 1–8) or without (lanes 9–16) metabolic labeling as in Fig. 2. Immunoprecipitations were done with either NRS (lanes 1, 3, 5, 7, 9, 11, 13, and 15) or polyclonal rabbit anti-Nef serum (α-Nef; lanes 2, 4, 6, 8, 10, 12, 14, and 16). An *in vitro* kinase assay was done on the immunoprecipitates from unlabeled extracts (lanes 9–16). Positions of the 62-kDa and 72-kDa phosphorylated substrates, CD8–Nef, and Nef are indicated at right. (*B*) The 62-kDa kinase substrate associated with native Nef is also phosphorylated on serine. Phosphoamino acid analyses were done as described for Fig. 2. (*C*) p62 from E-line and p62 from Jurkat cells are identical. The 62-kDa phosphorylated substrate (lanes 12 and 16 from *A*) was excised from the gel and treated with V8 protease as described.

phosphorylated protein were compared (Fig. 4*C*). The V8-protease patterns of these 62-kDa proteins from E-line and J.CN were identical. These results indicate that a common 62-kDa protein coimmunoprecipitates with Nef from T cells.

Truncated Nef Does Not Bind the Serine Kinase. During the course of selecting for high-level expression of the hybrid CD8–Nef protein in T-cell lines, a variant Jurkat cell line was established by cell sorting (A.B. *et al.*, unpublished work), which expresses a truncated form of CD8–Nef (J.CN.T). In these cells, the activation phenotype, which was observed with the full-length CD8–Nef, was lost (A.B. *et al.*, unpublished work). This truncated hybrid protein is ≈35 kDa (Fig. 5, lanes 5 and 6), and was found to have a metabolic half-life similar to that of the full-length hybrid CD8–Nef protein (data not shown). PCR analyses of *nef* sequences from J.CN.T cells revealed that the truncated protein is the result of single-nucleotide deletions leading to premature termination at amino acids 94 or 99 of Nef (A.B. *et al.*, unpublished work). When *in vitro* kinase assays were done on immunoprecipitates from J.CN.T extracts, neither the 62-kDa nor the 72-kDa phosphorylated proteins were detected (Fig. 5, lane 10). These results indicate that truncation of the full-length hybrid CD8–Nef protein abolishes the association of Nef with these phosphoproteins.

DISCUSSION

A cellular serine kinase activity has been found associated with Nef in stable T-cell lines constitutively expressing hybrid CD8–Nef proteins and most importantly in lymphocytes infected with two different HIV-1 isolates (HIV-1_{SF2}, HIV-1_{SF33}). Cellular proteins of 62 kDa and 72 kDa from both infected and CD8–Nef-expressing T lymphocytes were found to be phosphorylated on serine residues in *in vitro* kinase assays and to display similar partial V8-protease digestion patterns (Figs. 2 and 4). Thus, the hybrid CD8–Nef and wild-type Nef proteins are associated with the same cellular proteins. Because these phosphorylated proteins were not detected with the truncated hybrid CD8–Nef protein (Fig. 5), the C-terminal half of Nef must be important for the associated kinase activity.

Nef has been reported to have autophosphorylating or associated kinase activity (20, 22, 36). These previous studies, however, were not done with lymphocytes expressing Nef. In agreement with others (23, 24, 37, 38), results from our present study confirm the observation that Nef does not undergo autophosphorylation. Instead, Nef is associated with a kinase activity that phosphorylates other proteins. However, this activity is not due to PKC, PKA, or a tyrosine kinase (Fig. 3). The phosphorylation of Nef itself has been

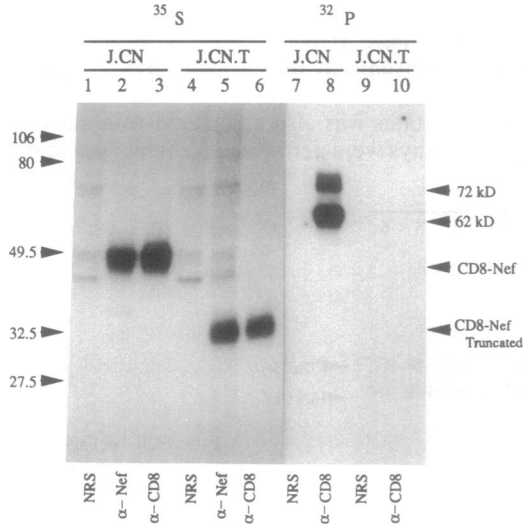

FIG. 5. Truncation of Nef results in loss of kinase activity. Jurkat cells expressing the hybrid CD8–Nef protein (J.CN) or a truncated form of the hybrid CD8–Nef protein (J.CN.T.) were extracted with (lanes 1–6) or without (lanes 7–10) metabolic labeling. Immunoprecipitations were done by using NRS (lanes 1, 4, 7, and 9), polyclonal anti-Nef serum (α-Nef; lanes 2 and 5), or anti-CD8 monoclonal antibody (α-CD8; lanes 3, 6, 8, and 10). Kinase assays were done on the unlabeled immunoprecipitates.

reported to be mediated by PKC (21). Therefore the kinase that phosphorylates Nef appears different from the kinase that associates with Nef.

At present, the function of the Nef-associated kinase is not known. Because previous studies indicated that Nef inhibits viral replication and long terminal repeat mediated gene expression *in vitro* (4–13), Nef could act by sequestering the kinase and preventing it from interacting with its normal target. Alternatively, the kinase itself could directly phosphorylate transcriptional regulatory molecules, such as IκB, which control viral gene expression. However, because there is a strong selection for full-length, functional *nef* genes in simian immunodeficiency-infected monkeys (18), the association of Nef with the kinase could have a positive effect on viral replication *in vivo*. In this regard, using the CD8–Nef system, high-level expression of Nef at the inner plasma membrane of lymphocytes results in T-cell activation, as manifested by expression of the T-cell activation markers CD69 and CD25 (A.B. *et al*, unpublished work). Moreover, truncations of Nef result in the loss of this activated phenotype. Thus, a correlation exists between the ability of Nef to bind the cellular serine kinase and its ability to activate T cells when this viral protein is expressed at the plasma membrane. These results, therefore, suggest that Nef affects the signal-transduction cascade.

Thus far, the kinase associated with Nef has not been identified, and the identities of the 62-kDa and 72-kDa phosphorylated proteins are currently unknown. It is conceivable that the 62-kDa or the 72-kDa phosphorylated protein is the kinase(s) that autophosphorylates in the *in vitro* kinase assay. However, it is also possible that the kinase itself does not autophosphorylate, as is the case with cdc2 kinase (39), and the phosphorylated proteins could simply represent kinase substrates. In an attempt to identify the kinase that associates with Nef, immunoblot analyses with antibodies to known serine kinases and other signal-transducing molecules have been performed; antibodies against Raf, S6 kinase, GTP-associated protein (GAP), and MAP kinase do not react with any of the proteins associated with Nef. Furthermore, neither anti-lck nor anti-CD4 antibody recognized the 62-kDa or 72-kDa protein (E.T.S. and A.B., unpublished data). These findings suggest that the 62-kDa and 72-kDa proteins are distinct from the cellular components known to be associated with CD4 expression on the cell surface. Our observations, therefore, suggest that the kinase with which Nef associates may be distinctive. Identification of the kinase and characterization of its substrates may reveal the pathway by which Nef functions in T cells.

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Note. Recently, another report describing the association of cellular proteins with Nef has been published (40).

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