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SIGNALS TRANSDUCED THROUGH THE CD4 MOLECULE ON T LYMPHOCYTES ACTIVATE NF-κB

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We have demonstrated that native envelope glycoproteins of HIV-1, gp160 can induce activation of the transcription factor, NF-κB. The stimulatory effects of gp160 are mediated through the CD4 molecule, since pretreatment with soluble CD4 abrogates its activity. The gp160-induced NF-κB complex consists of p65, p50 and *c-rel* proteins. The stimulatory effect of gp160 on NF-κB activation is protein synthesis independent, is dependent upon protein tyrosine phosphorylation, and abrogated by inhibitors of protein kinase C. The gp160-mediated activation of NF-κB in CD4 positive T cells may be involved in biological effects, e.g.,enhanced HIV replication, hypergammaglobulinemia, increased cytokine secretion, hypercellularity in bone marrow and apoptosis.

Signals transduced through the CD4 molecule have been shown to play an important role in T cell activation (1). The biochemical nature of these signals has however been controversial; while increase in intracellular calcium, hydrolysis of phosphatidyl inositol (PI) and activation of tyrosine kinases have been demonstrated by some (2-5), others failed to observe these events (6,7). The cytoplasmic tail of the CD4 molecule on T lymphocytes is noncovalently associated with the *src*-homology tyrosine kinase, $p56^{lck}$ (8); in addition, a GTP-binding protein associated with the CD4-T cell receptor complex (9) has been shown to play an important role in the transduction of CD4-mediated signals. The CD4-mediated signals have recently been shown to involve activation of a *raf*-1-related 110 kD polypeptide and PI-3 and PI-4 kinases (10,11). In this study, utilizing HIV envelope glycoproteins and anti-CD4 mAb, we have demonstrated CD4-mediated signals result in activation of the Nuclear factor -κB (NF-κB) in CD4+ T cells.

NF-κB, originally identified as a transcriptional activator for the immunoglobulin κ-light chain (12), has been implicated in transcriptional control of a variety of genes e.g. IL-2, IL-2 receptor and the HIV LTR (13). The expression of NF-κB can be triggered by multiple stimulants including phorbol esters, TNF-α, tax protein of HTLV-1 and ultraviolet radiation, by post-translational mechanisms involving phosphorylation-induced degradation of the cytoplasmic inhibitor protein, IκB (14). Several proteins have been indentified in the NF-κB/rel family of transcription factors which include v-rel and its cellular gene *c-rel* (15), Xrel1 (16), relB (17), the

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subunits of NF-κB complex, NFKB1 p110/p50 (18), RelA/p65 (19), NFKB2 p105/p52 (20) and the *Dorsal* gene of Drosophila morphogen (21). These proteins induce transactivation by direct interaction with a decameric 5' GGGACTTTCC 3' DNA sequence motif (22). Direct interaction of *c-rel* with TATA-binding protein, TF-IID and NF-κB p50 to TF-IIB (23,24) in activated cells has been shown to lead to enhanced transcriptional activation by NF-κB. Utilizing envelope glycoproteins of HIV and anti-CD4 mAbs, we have investigated the role of the signals mediated through the CD4 molecule in T cells resulting in activation of NF-κB.

MATERIALS AND METHODS

Antibodies and reagents. Polyclonal rabbit anti-gp120 was developed by Advanced BioScience Labs Inc.; rabbit anti-human p65 (1226, against the C-terminal aminoacids 527-550), p50 (1141, against the N-terminal aminoacids 2-15) and *c-rel* (265, against the C-terminal aminoacids 573-587) were gift from Dr. Nancy Rice, Frederick, MD; soluble CD4 was a gift from Genentech, San Fransisco, CA; Herbimycin A, H-7, cycloheximide, verapamil, cyclosporine A were purchased.

Envelope glycoproteins. Native gp160 and gp120 were purified by affinity chromarography from a clone of Hut-78 cells (6D5), as described earlier (26). The two proteins were >95% pure and were not contaminated with endotoxins as tested by the Limulus amoebocyte test kit (Sigma).

Cells: CD4 positive clone of Jurkat T cells, E6-1, obtained from ATCC, Bethesda, MD (25), was maintained in RPMI 1640 media (Whittaker) supplemented with pennicillin and streptomycin and 10% FCS. CD4 positive T cells H9, Molt 4 were purchased from ATCC, Betheda, MD. CD4 negative Jurkat T cells (JN) were mutant CD4 negative cells by FACS analysis.

Perpheral blood lymphocytes (PBL) were purified by ficoll-hypaque density gradient centrifugation as described earlier (27). T cells were purified from PBL by rosetting 2x with neuraminidase treated sheep RBC as described earlier (27).

Immunomagnetic separation of $CD4^+$ and $CD8^+$ T cells. Purified T cells stimulated with medium alone or with 1μg/ml gp160 for 4 hours were incubated with anti-CD8 immunomagnetic beads (Dynal, Great Neck, NY) for 30 minutes at 4°C on a rotating shaker, as recommended by the manufacturer. The cells were subjected to a magnetic field, and the unbound cells (designated CD8 negative, CD8- T cells) were carefully aspirated. The bound cells were designated CD8 positive ($CD8^+$). Nuclear proteins were extracted as described below.

Nuclear extracts. Small scale nuclear extracts were made from 2×10^7 unactivated or activated T cells as described (28). Unless otherwise stated, cells were stimulated with medium alone or various stimuli for 4 hours at 37°C. Cells were washed an resuspended in 10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ 0.5 mM dithiothreitol and 0.5 mM PMSF and lysed by the addition of Nonidet p40 to a final concentration of 0.5%. Nuclei were pelleted and washed in the same buffer without Nonidet p40, and nuclear proteins extracted in buffer C (29). After pelleting nuclear debris, the supernatant was removed and diluted with an equal volume of buffer D (29). This extract was used directly in the EMSA. The equivalance of the extracts were verified by protein estimation using the BCA protein kit (Pierce Chemical Co., Rockford, IL). For some experiments, nuclear extracts were preincubated with antibodies to p65, p50, *c-rel* or normal rabbit serum for 1 hour on ice. While the antibody-treated nuclear extracts were electrophoresed directly in some experiments, in others, the immune complexes were precipitated with Sepharose-conjugated Protein A (Pharmacia) in others.

Electromobility shift assays (EMSA). Consensus NF-κB oligonucleotides were obtained commercially (Promega) and end-labelled with ^{32}P γ-ATP and polynucleotide kinase. For each binding reaction, 10,000 cpm (~0.2-0.5 ng) of end-labelled oligonucleotide was incubated for 30 minutes at room temperature with 5-8 ug of nuclear extract in the presence of 3 μg sheared poly dI-dC (Pharmacia). The resulting DNA-protein complexes were analysed by electrophoresis at 4°C of 4% polyacrylamide gels. Unlabelled oligonucleotides used for competitions were added to nuclear extracts and dI-dC prior to addition of labelled probe.

RESULTS

Gp160 induced activation of NF-κB in CD4$^+$ T cells. *Figure 1* shows that the basal level of NF-κB activation in the CD4$^+$ E6-1 cells could be enhanced by stimulation with gp160 in a dose dependent manner. gp120, anti-CD4 mAb (Leu3a) and PMA alone could also induce NF-κB activation in the CD4$^+$ E6-1 cells. Specific binding was demonstrated by the addition of excess unlabelled NF-κB oligonucleotides (competitor). The gp160-induced NF-κB binding was observed within 30 minutes of stimulation with gp160, peaked at 4 hours, persisted upto 24 hours (data not shown).

The gp160-induced NF-κB complex comprised of p50, p65 and *c-rel* proteins. *Figure 2* shows that addition of these antibodies against p65, p50 and *c-rel* proteins induced a supershift in EMSA; no supershift was observed in nuclear extracts treated with normal rabbit serum. In order to confirm the observation that these proteins were involved in the protein-DNA interactions, nuclear extracts were first treated with specific antibodies and immune complexes cleared by addition of Sepharose-Protein A beads. The gp160-induced NF-κB could be partially abrogated by antibodies to p65, p50 and *c-rel* in a dose dependent manner; here again normal rabbit serum had no effect (data not shown). Although these observations suggest that the gp160-induced NF-

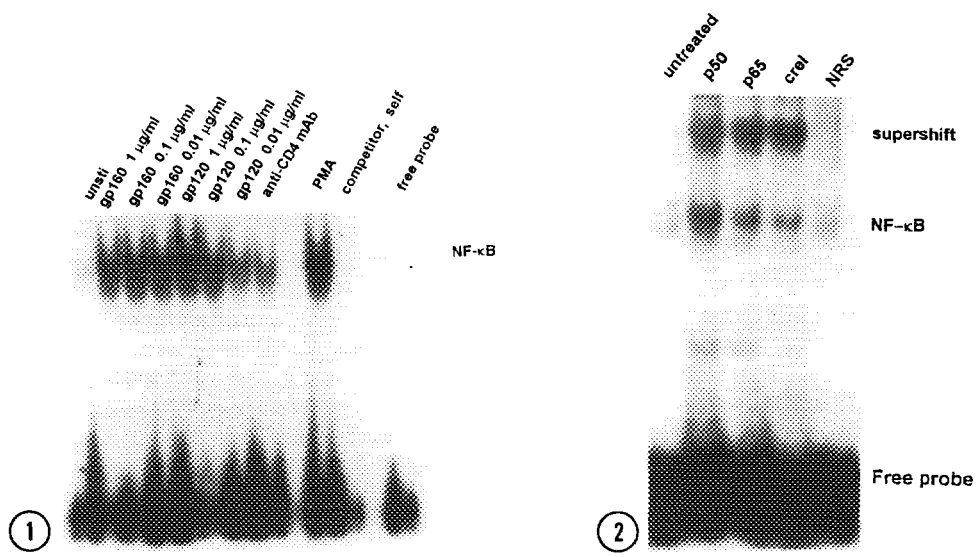

Figure 1. Stimulation of CD4 positive T cells with gp160 induces NF-κB activation: Stimulation of E6-1 cells was carried out by addition of medium alone gp160 and gp120 or anti-CD4 mAb (Leu3a) or 50 ng/ml phorbol myristate acetate (PMA), for 4 hours at 37°C; the first lane comprised of free probe, and the last lane, competition of NF-κB binding by 10x cold NF-κB oligonucleotides. All the results are a representative of at least 5 separate experiments.

Figure 2. Addition of antibodies to p65, p50 and *c-rel* induces supershift of the NF-κB binding: E6-1 cells were stimulated with 1 µg/ml gp160 for 4 hours at 37°C. Nuclear extracts were incubated with 1 µl of antibody to p65, p50, *c-rel*, 1:5 normal rabbit serum, followed by incubation with ^{32}P-labelled NF-κB oligonucleotides, and poly-dI dC as indicated in methods. Supershift in the EMSA suggests binding of the antibodies to NF-κB.

κB comprised of p65, p50 and *c-rel*, it should be noted that our experiments do not define the nature of the complexes (homo- or heterodimer forms).

The gp160-induced activation of NF-κB was mediated through the CD4 molecule. *Figure 3* shows that the stimulatory activity of gp160 on E6-1 cells could be abrogated by pretreatment of gp160 with soluble CD4. To further demonstrate that gp160 induced NF-κB activation through the CD4 molecule, $CD4^+$ and CD4 negative T cell lines were analysed. *Figure 4* shows that gp160 could stimulate NF-κB activation in $CD4^+$ H9 cells, Molt4 cells, but not in CD4 negative mutant Jurkat T cells (JN). Here again, pretreatment of gp160 with soluble CD4 abrogated NF-κB activation in $CD4^+$ T cells. All these cells could be effectively induce NF-κB activation upon stimulation with PMA. These results strongly demonstrate that gp160 mediates its stimulatory activity of NF-κB activation through the CD4 molecule.

Gp160 induced NF-κB activation in peripheral blood $CD4^+$ T cells. In order to determine whether gp160 could activate normal physiological T cells, purified peripheral blood T cells were stimulated with gp160 for 4 hours. $CD4^+$ T cells were separated from $CD8^+$ T cells by negative selection using anti-CD8 mAb-conjugated magnetic beads. *Figure 5* shows that stimulation of purified T cells with gp160 induced activation of NF-κB in CD8- T cells (>90% $CD4^+$ by flow cytometry), but not in $CD8^+$ T cells. [Only non-specific binding was observed in $CD8^+$ T cells, which could not be abrogated by competition with excess unlabelled oligonucleotides]. The stimulatory effects of gp160 could be abrogated by pretreatment of the gp160 with soluble CD4 (data not shown). Soluble CD4 itself did not induce activation of NF-κB.

Signal requirements for the gp160-induced NF-κB activation. In order to investigate the nature of the signals involved in the activation of NF-κB by gp160, several pharmacological inhibitors were utilized. *Figure 6* shows that addition of Herbimycin A (HA, inhibitor of tyrosine phosphorylation), and H-7 (inhibitor of protein kinase C) abrogated gp160-induced NF-κB activation. Cyclosporine A (CsA) and the protein synthesis inhibitor, cycloheximide (CHX) had no significant effect on activation of NF-κB, as did the calcium channel blocker, verapamil (ver) [data not shown]. These studies indicate that tyrosine phosphorylation, and activation of protein kinase C were involved in the mechanism of the gp160-induced activation of NF-κB.

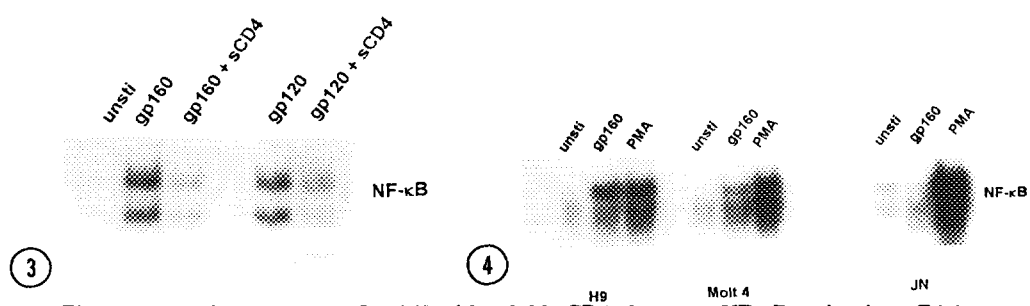

Figure 3. Pretreatment of gp160 with soluble CD4 abrogates NF-κB activation: E6-1 cells were stimulated with medium alone or 1 μg/ml gp160 in the presence of 10, 1 μg/ml of soluble CD4 (Genentech, CA). EMSA were performed as described in methods.

Figure 4. CD4 positive T cell lines, but not CD4 negative T cell lines,could be induced by gp160 to increase NF-κB activation. H9 cells Molt4 cells or JN cells were stimulated with medium alone,1 μg/ml gp160, or PMA. EMSA were performed as described in methods.

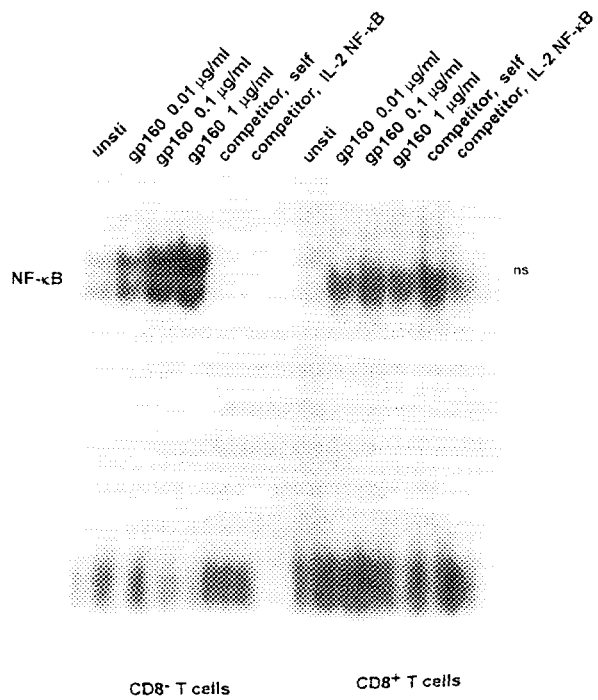

Figure 5. gp160 can induce $CD4^+$ but not $CD8^+$peripheral blood T cells to induce NF-κB activation. Purified T cells were stimulated with medium alone, gp160 and gp120 . CD4 and CD8 positive T cells were separated by anti-CD8 mAb-conjugated magnetic beads (Dynal, Great Neck, NY). Adherent cells were denoted CD8 positive and non-adherent cells as CD4 positive. Nuclear extracts were assessed for NF-κB binding by EMSA.

DISCUSSION

To the best of our knowledge, this is the first study which demonstrates activation of NF-κB in human T cells by signals transduced directly through the CD4 molecule.

Activation of T cells through the cell surface receptor results in a cascade of biochemical events which lead to gene transcription (30,31). NF-κB is a family of transcription factors that

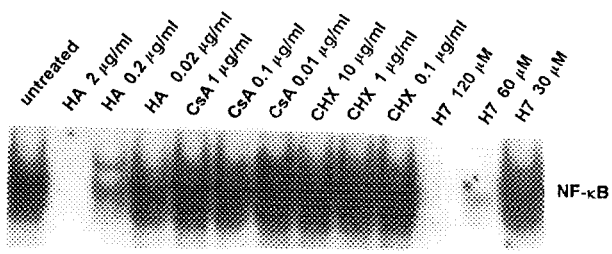

Figure 6. The gp160-mediated NF-κB binding is dependent on tyrosine phosphorylation, activation of protein kinase C, but not on protein synthesis, increase in intracellular calcium or CsA: E6-1 cells were stimulated with 1 µg/ml gp160 for various time intervals in the absence or presence of cycloheximide [CHX], cyclosporine A [CsA], Herbimycin A [HA], or H7. EMSA were performed as described in figure 1.

bind to a distinct DNA sequence, and have been shown to co-operate in potentiating biolgical activities (22). In this study we have demonstrated that binding of gp160 to $CD4^+$ T cells results in activation of NF-κB. The stimulatory effects of gp160 are mediated through the CD4 molecule, since pretreatment of gp160 with soluble CD4 abrogates its activity. Furthermore, cell lines expressing the CD4 molecule (H9, Molt4), but not CD4 negative cell line (JN) can be induced by gp160 to activate NF-κB. gp160 can also stimulate peripheral blood $CD4^+$ cells, but not CD8 positive T cells to activate NF-κB. Finally, the stimulatory effects of gp160 can be mimicked by anti-CD4 mAb. These results clearly demonstrate that gp160 can induce NF-κB activation by direct stimulation through the CD4 molecule.

Various combinations of the NF-κB family members have been shown to be involved in homo- and heterotypic dimerizations, which result in positive or negative regulation of transactivation by the κB -controlled reporter genes in transient transfection assays (15-21). The gp160-induced NF-κB complex comprises of at least p65, p50 and *c-rel* proteins, since pretreatment of the nuclear extracts with specific antibodies to these proteins could abrogate NF-κB binding. Previous studies have demonstrated that the NF-κB complex in resting $CD4^+$ T cells consists of p50-p50 homodimers, which upon activation, form p65-p50 heterodimers and induce κB transctivation in Th1 (32). The understanding of the nature of the proteins in the NF-κB complex in the gp160-stimulated $CD4^+$ T cells may give an insight into the regulatory role of CD4-mediated signals in T cell activation.

Since the protein synthesis inhibitor, CHX failed to abrogate NF-κB binding, the mechanism of the gp160-induced activation of NF-κB occurs by post-translational modification of pre-existing NF-κB complex. Post-translational modification upon T cell activation involve phoshorylation of IκB which results in its degradation, thus releasing the active NF-κB complex to translocate to the nucleus and induce transactivation (14). Phosphorylation of IκB has been shown to be mediated by PKC and *raf*-1 kinase (12,33). In our study, addition of PKC inhibitor, H-7, and inhibitor of tyrosine kinases, herbimycin A, abrogated the gp60-induced NF-κB activation. Intracellular calcium channel blocker, verapamil failed to inhibit NF-κB activation, suggesting Ca^{++} is not directly involved in this activation pathway. Calcineurin, the phosphatase which modulates NFAT activity (34), is not involved in the gp160-induced NF-κB activation, since its activity was unaffected by cyclosporine A. These observations suggest that activation of PKC, and tyrosine kinase activity by gp160/gp120/anti-CD4 mAbs through the CD4 molecule may be involved in the mechanism of NF-κB activation in T cells.

Given that the promoters of IL-2, IL-3, GM-CSF and the HIV-LTR contain NF-κB binding sites, it is possible that the gp160-induced activation of NF-κB in T cells can regulate the expression of these molecules. In this respect, we have demonstrated that the direct stimulatory effects of gp160 result in normal $CD4^+$ T cell clones to secrete IL-6 and upregulate CD40 ligand, resulting in polyclonal B cell differentiation (35), and cord blood T cells to secrete IL3, IL-6 and GM-CSF, inducing *in vitro* differentiation of myeloid progenitos in cord blood (36,37). gp160 has also been shown to prime T cells to undergo apoptosis (38). It is possible that gp160-induced NF-κB activation may play a significant role in these biological effects.

In conclusion, we have demonstrated that soluble envelope glycoproteins of HIV-1, gp160, by binding to the CD4 molecule on T cells, may transduce signals which result in NF-κB activation which may be contribute to pathogenesis of HIV infection.

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