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Nucleotide and Amino Acid Homology Between the Human Thyrotropin Receptor and the HIV-1 Nef Protein: Identification and Functional Analysis†

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A comparison of the nucleotide sequence of the human thyrotropin receptor (hTSH-R)with that of HIV-1 revealed 61% homology between a 161 base pair region encoding a unique portion of the hTSH-R and an immunogenic HIV-1 regulatory protein, nef. Amino acid analysis of this region shows 27% homology, including a segment in which 7/10 consecutive amino acids are identical. Sera from rabbits successfully immunized with a 16 amino acid portion of the hTSH-R (352-367, p1) was assessed for reactivity against a partially homologous nef peptide (nef-1) by ELISA, with a finding five-fold higher post-immunization values compared to pre-immune sera. The specificity of this response was verified with Western blot, using recombinant nef protein. An ELISA using nef-1 gave 64% higher values with sera from Graves' disease patients than with normal controls. This homology and immunologic cross-reactivity suggests an avenue through which a shared immune response against an HIV-1 related retrovirus could play a role in the pathogenesis of Graves' disease.

The recent identification and sequencing of the cDNA for the human thyrotropin receptor (1, 2, 3) has allowed rapid progress in the study of the immune response against this central autoantigen in Graves' disease and idiopathic hypothyroidism. One focus of investigation has been the localization of antigenic epitopes within this molecule. A region of particular interest is a 51 amino acid segment (333-384) of the extracellular domain in which the receptor diverges widely from the otherwise homologous hLH/CG receptor. This portion of the molecule is predicted to be hydrophilic and hence exposed on the surface of the protein in vivo. While apparently unimportant for thyrotropin binding or transduction of signal (4), this segment has been found to be highly antigenic in laboratory animals (5, 6) and contains a domain which is apparently recognized by 80% of patients with Graves' disease (7).

†The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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A viral role in the pathogenesis of autoimmune disease in general has long been an alluring area of speculation although conclusive evidence for this involvement in humans is lacking (8). Possible mechanisms for viral mediation of autoimmune disease include polyclonal B-cell and T-cell activation with perhaps subsequent release of cytokines, induction of aberrant MHC class I and II antigen expression on target tissue cell membranes, release of previously sequestered host antigens through direct cell damage, modification of host antigens resulting in altered immunogenicity, and molecular mimicry resulting in cross-reactivity between anti-viral immune effectors and host antigens.

Due to the potential importance of the 51 amino acid unique region of the hTSH-R in the antigenic response against this protein, we performed a directed computer-based homology analysis of the viral nucleotide database to detect possible substrates for molecular mimicry. The unexpected finding of extensive nucleotide and partial amino acid homology with the HIV-1 nef protein is described herein as well as an evaluation of the functional nature of this homology in terms of immune cross-reactivity.

MATERIALS AND METHODS

Computerized Homology Search: Computerized nucleotide homology search of GenBank (9) using the International Biotechnologies Inc. (IBI) software program was performed on the unique region of the hTSH-R between base pairs 1096 and 1251 (the numbering system corresponds to that of reference 1) as well as adjacent areas.
Source of peptides and recombinant nef protein: The hTSH-R peptide, p1, representing amino acids 352-367 was the generous gift of Dr. Leonard Kohn. A nef peptide representing amino acids 49-66 (nef-1) was purchased from Peptide Technologies Corp., Washington, D.C. Recombinant nef protein from the HIV-1 BH10 strain was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.
Source of sera: Two NZW rabbits were immunized intradermally with p1, dispersed in Freund's complete adjuvant. Immunizations utilized 40.0 ug of peptide. The primary immunization was followed by two booster immunizations at 2 and 4 weeks, respectively. Blood was obtained through ear vein cannulation on days 0, 21, 28, 56, and 84. The success of the immunization was verified by ELISA as described elsewhere (10). After obtaining informed consent in a hospital approved protocol, blood was obtained from ten patients with Graves' disease as well as ten normal volunteers. Two types of control anti-nef sera were utilized. A mouse monoclonal antibody (No. NF2-B2) formed against an N-terminus epitope in recombinant nef from the BH10 strain was provided by the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (11). In addition, polyclonal rabbit sera against amino acids 2-28 of nef from the HXB-3 strain of HIV-1 was obtained from the above agency donated by Dr. Bryan Cullen (12).
ELISA: Immulon ELISA plates (Dynatech, Chantilly, VA.) were coated with 20 ug/ml of nef 49-66 in carbonate-bicarbonate buffer at pH 9.6 at 4 °C for 16 hours. After blocking with 1% BSA, sera were added at a dilution of 1: 50 and incubated for 2 hrs at 37 °C. Alkaline phosphatase conjugated anti-human, anti-rabbit, or anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was subsequently added at a dilution of 1:1000 and incubated at 37 °C for one hour. Washing steps utilized PBS with 0.5% Tween-20. BCIP/NBT was used as substrate. Serial optical densities were measured at 405 nm on an ELISA plate reader. All samples were run in duplicate and mean values reported.
SDS-PAGE and Western blot Analysis: SDS-PAGE was performed under denaturing conditions using 30.0 ug/ lane of recombinant nef protein from BH10 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) on a 10-20 % polyacrylamide gel. Electrotransfer to Immobilon PVDF membranes (Millipore Corp., Bedford, MA), was performed at 4 °C over 12 hours. After blocking with 1 %

gelatin, membranes were exposed to sera diluted 1:100 (human) or 1:200 (rabbit, mouse) at room temp for two hours. Alkaline phosphatase conjugated second antibody (as per ELISA description) was added at a 1:1000 dilution for one hour at room temperature. p-Nitrophenyl phosphate (Sigma, St. Louis, MO.) was used as substrate following dilution as per the manufacturer's recommendations.

RESULTS

Computerized Homology Search: Computerized homology search of the nucleotide database of GenBank revealed a 161 base pair region of the human thyrotropin receptor (1090-1250) in which 61% homology occurred with an isolate of HIV-1 nef. Fig. 1 shows the nucleotide sequence of this region of the hTSH-R aligned with the published sequences of the nef encoding portion from four different HIV-1 species. There are regional differences in the extent of homology in this segment of the gene with variation from as low as 50% homology in 20 contiguous bases between 1090-1109 to as high as

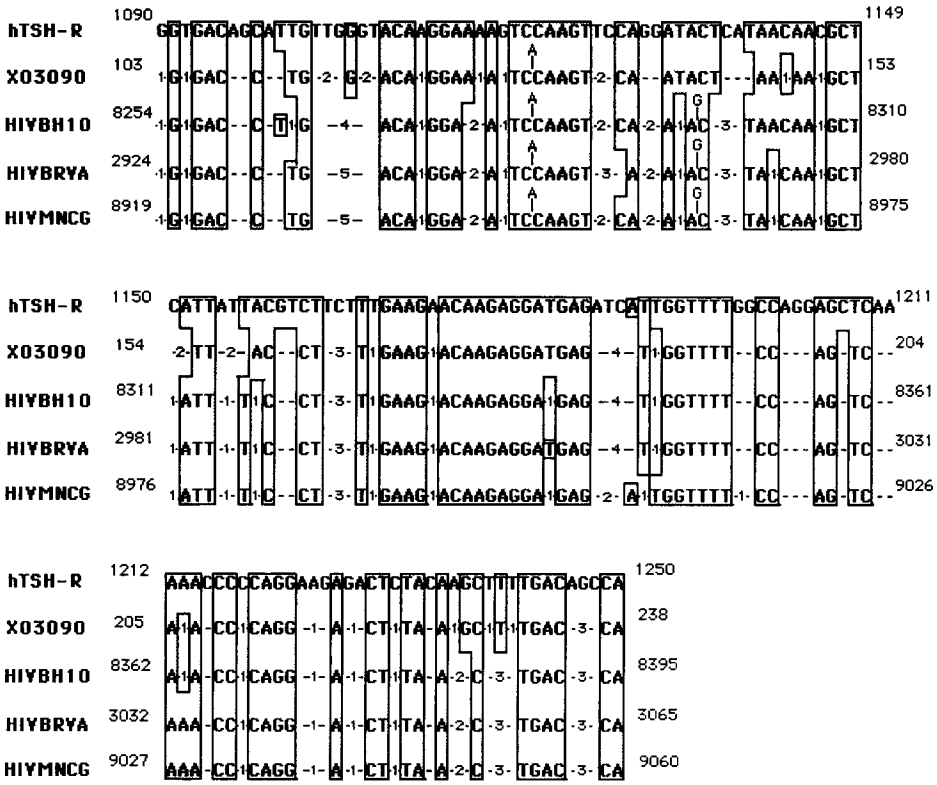


Fig. 1 Comparison of the nucleotide sequence of hTSH-R to four different nef species. Gaps are represented as (- -), variation from hTSH-R are shown as (- n -) where n is the number of consecutive mismatched bases. Areas of adjacent gap and nucleotide mismatch occur when n is less than the number of corresponding bases in hTSH-R. X03190 is the GenBank number for isolate HAT3 from Ratner et al. (1985), Nucleic Acids Research, 13, 8219-8229; HIVBH10 is from Ratner et al. (1985), Nature, 313, 277-284; HIVBRVA is from Anand et al., (1989), Virology, 168, 79-89; and HIVMNCG is from Gurgo et al., (1988), Virology, 164, 531-536.

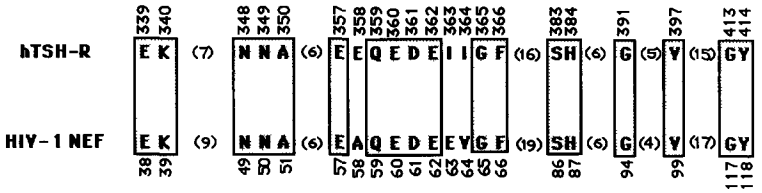
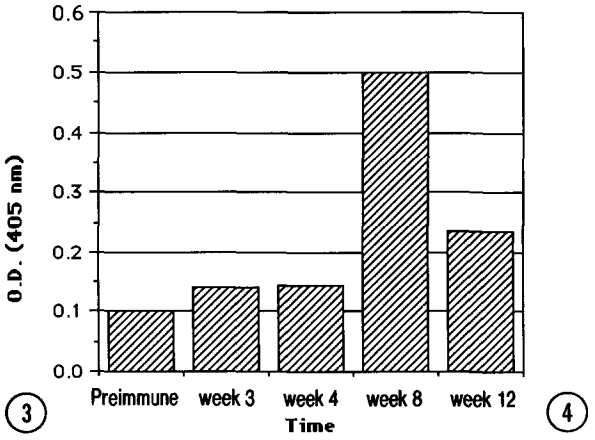


Fig. 2 The amino acid sequence for hTSH-R is aligned with the region of homology with the HIV-1 nef protein, HAT-3 isolate. The numbers in parentheses represent the number of consecutive non-homologous amino acids.

90% homology in a similar sized region from 1167-1186. Amino acid comparison with the HIV-1 nef isolate with the highest extent of homology is shown in Fig. 2. It can be seen that 14 of 51 (27%) amino acids in the unique region of the hTSH-R (amino acids 333-384) are homologous with the nef protein. Of particular interest is a focal region of 70% amino acid homology within the particularly hydrophilic segment of the hTSH-R from amino acid 357-366. This homology continues to a lesser extent in the N-terminal direction beyond the unique region of the hTSH-R. The entire region of homology is contained within the tenth exon of the hTSH-R gene (13).

Reactivity of sera from immunized rabbits: One of two rabbits (rabbit #1) immunized with hTSH-R peptide, p1, developed a high titer anti-p1 immunoreactivity as seen by ELISA and Western blot technique (10). The second rabbit (rabbit #2) had an initially low



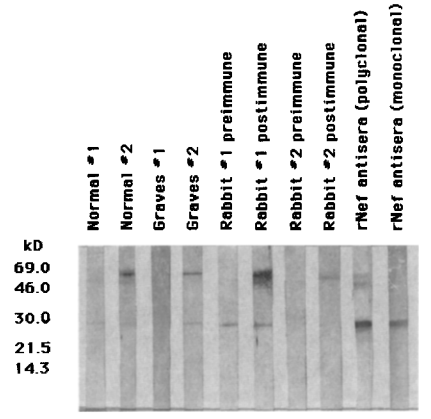


Fig. 3 Serial immunoreactivity against nef-1 using pre- and postimmune sera from a rabbit immunized against the hTSH-R peptide, p1. Sera were diluted 1:50 before use.

Fig. 4 Western blot of recombinant nef protein using Graves' and normal sera as well as pre- and postimmune rabbit sera following immunization with the hTSH-R peptide, p1, and control nef antisera. rNef protein is seen in monomeric (27 kD) and dimeric (55 kD) forms.

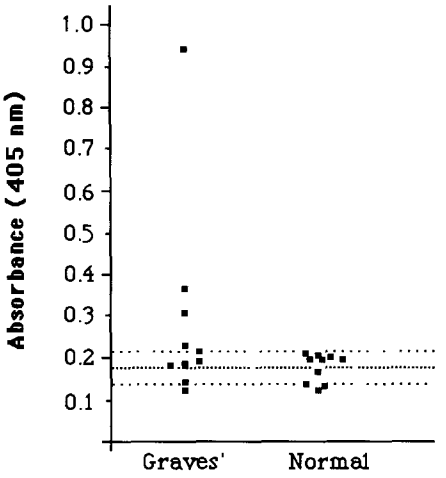


Fig. 5 Immunoreactivity of Graves' and normal sera at a 1:50 dilution against nef-1. The dotted lines represent the mean ± 2 SD for the normal sera.

antibody titer against this peptide which subsequently increased. Fig. 3 demonstrates the cross-reactivity of the immune response against p1 with the partially homologous nef peptide, nef-1. A five-fold O.D. (405 nm) increase at 8 weeks following immunization with p1 was demonstrated for rabbit #1. Similar data for the second rabbit showed no significant increase. The specificity of the response was verified with Western blotting techniques against the full-length nef protein as shown in Fig. 4. The recombinant nef protein as prepared by the AIDS Reference Reagent Program contributor is > 95% pure and present in both dimeric (55 kD) and monomeric (27 kD) forms as seen by Coomassie blue staining. Preimmune sera from rabbit #1 shows only slight binding to the monomeric form of nef compared to an intense reactivity, particularly with the dimeric form of the molecule, when using postimmune serum. As anticipated, a less dramatic response was seen in the second rabbit with the poor early response to immunization by ELISA. Control sera binding on Western blot show binding to primarily the monomeric form by the mouse anti-nef monoclonal antibody, whereas the polyclonal rabbit antisera recognized both forms of nef.

Graves' disease patients and normal volunteer immunoreactivity against nef-1: Fig. 5 demonstrates ELISA readings against nef-1 using sera from ten patients with Graves' disease and ten normal controls. Although the mean ELISA value from patients with Graves' disease was 64% higher than those obtained in normal volunteers, this difference failed to attain significance. It is notable, however, that three patients with Graves' disease had ELISA values greater than 3.5 standard deviations above the mean value obtained in the normal volunteers. In contrast, limited Western blot analysis of sera from two Graves' disease patients and two normal controls using full-length recombinant nef protein (fig. 4) showed a similar pattern of reactivity with one normal and one Graves' disease patient each binding to both the monomeric and dimeric forms of nef.

DISCUSSION

In this study, we describe nucleotide and amino acid homology between a unique region of the human thyrotropin receptor and the HIV-1 nef protein. This homology is greater at the nucleotide than amino acid level, yet certain amino acids appear to be highly conserved, despite the rapid rate of genetic drift previously shown in the HIV-1 genome (14). The homology occurs in a segment of the hTSH-R calculated to be hydrophilic based on consideration of the constitutive amino acids; prior experimental evidence has shown this region to be antigenic in vivo (5, 6). Significantly, the segment of greatest homology with nef coincides with an hTSH-R peptide which has recently been shown to be recognized by sera from 80 % of patients with Graves' disease (7).

Our findings of postimmune rabbit sera recognition of nef-1 following successful immunization with the partially homologous hTSH-R peptide, as seen by ELISA and Western blot techniques, demonstrates immune cross-reactivity between these two proteins. It is of interest that, by Western blot, the dimeric form of nef appears to have a higher immunoreactivity than the monomeric form of this protein although the explanation for this finding is unclear. We next sought to determine whether this cross-reactivity might extend to patients with Graves' disease with known immunoreactivity against the hTSH-R. Although there was a wide range of immunoreactivity against nef-1 in the Graves' patients tested, three patients had marked elevation against this peptide suggesting a prior exposure to an antigenically similar protein. However, the two Graves' patients tested against full-length recombinant nef by Western blot failed to show a dramatically enhanced banding pattern compared to the two normal controls. Several possible explanations exist for the apparent discrepancy between the ELISA and Western blot findings in these patients. One explanation is that rather than indicating a prior exposure to nef, the ELISA results reflect the previously noted high prevalence of immunoreactivity against p1 in Graves' disease, coupled with the immune cross-reactivity between p1 and nef-1 described in the rabbit data in this study. Another possibility is that the ELISA data using Graves' sera represented a nonspecific, enhanced immunoreactivity in these individuals rather than a specific response against nef. This latter possibility seems unlikely in view of the extensive washing steps with detergent utilized throughout the procedure to reduce nonspecific binding. A last possibility is that the reducing conditions under which the PAGE was performed may have altered the conformation of nef sufficiently to prevent immune recognition by anti-nef IgG, thereby altering the Western blot findings.

The function of nef in HIV-1 and related lentiviruses is unknown, although it is believed to exert a regulatory influence in the expression of the virus particle. The exact nature of this regulatory function remains controversial (13, 15). Of significance to the current study, the nef protein is known to be the focus of an early immune response in HIV-1 infected individuals, often predating the establishment of the classic antibodies used in the serologic diagnosis of HIV-1 infection (16). In addition, certain control patients with common dermatologic conditions at low risk for HIV-1 infection have been found to have an antibodies against nef, leading the authors to speculate that these 'early' antibodies

may in some cases actually reflect cross-reactivity to an unrelated normal cellular element (17). In previous studies designed to localize important immunogenic regions of the nef protein, two highly antigenic segments have been identified (18), one of which (amino acids 45-69) coincides with the region of highest amino acid homology with the hTSH-R as identified in the current study. The authors state that this antigenicity could prove useful in the development of effective anti-HIV-1 vaccines, resulting in destruction of infected cells at an early stage. Our findings of immune cross-reactivity between nef and the hTSH-R could theoretically impinge on the utility of such an approach.

Increasing evidence is accumulating for a viral role in the pathogenesis of autoimmune disease (8). A recent study has demonstrated antibodies to the HIV-1 gag p24 protein in 22 of 61 (36%) of patients with systemic lupus erythematosus who were without other evidence of HIV-1 infection (19). A specific endogenous avian leukosis retrovirus has been identified in the obese strain chicken model of spontaneous autoimmune thyroiditis (20). A study of salivary tissue from patients afflicted with Sjögrens syndrome has revealed the presence of a retrovirus which is antigenicity related (but not identical) to HIV-1 in 2 of 6 individuals studied (21). In addition to the previously cited mechanisms through which viral infection may contribute to autoimmunity, our data suggest an avenue through which, in a permissive genetic and immunologic milieu, exposure to an HIV-1 related lentivirus could trigger an autoimmune response against the hTSH-R. It should be emphasized that the data presented, while providing the first published evidence of an amino acid homology and immune cross-reactivity between a retroviral antigen and the hTSH-R, has shown no definitive evidence of a retroviral association with Graves' disease. The significance of these findings in terms of understanding the pathogenesis of Graves' disease, therefore, remains speculative at present.

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