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Hydrogen exchange mass spectrometry of related proteins with divergent sequences: A comparative study of HIV-1 Nef allelic variants

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Abstract

Hydrogen exchange mass spectrometry can be used to compare the conformation and dynamics of proteins that are similar in tertiary structure. If relative deuterium levels are measured, differences in sequence, deuterium forward- and back-exchange, peptide retention time and protease digestion patterns all complicate the data analysis. We illustrate what can be learned from such data sets by analyzing five variants (Consensus G2E, SF2, NL4-3, ELI, and LTNP4) of the HIV-1 Nef protein, both alone and when bound to the human Hck SH3 domain. Regions with similar sequence could be compared between variants. While much of the hydrogen exchange features were preserved across the five proteins, the kinetics of Nef binding to Hck SH3 were not the same. These observations may be related to biological function, particularly for ELI Nef where we also observed an impaired ability to downregulate CD4 surface presentation. The data illustrate some of the caveats that must be considered for comparison experiments and provide a framework for investigations of other protein relatives, families, and superfamilies with HX MS.

Graphical Abstract

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HX MS of variants

Keywords

Hck SH3; protein conformation; sequence conservation

INTRODUCTION

Vish Katta and Brian Chait published [1] the first descriptions of direct detection of hydrogen/deuterium exchange (HX) in proteins by mass spectrometry (MS). The 1991 Katta and Chait paper is required reading in the HX MS field and was, in many ways, ahead of its time. In the ensuing 25 years, many types of proteins have been analyzed with HX MS, ranging from small and simple proteins (e.g. insulin) all the way to large, multi-protein complexes. Technology derived from Brian Chait's first studies has made complex analyses faster, more reliable and robust, and has enabled comparative analysis of related proteins. Our laboratory has been interested in using HX MS to compare proteins that are related in tertiary structure, but not necessarily in primary structure (e.g., [2,3]). In undertaking these types of homologous protein comparison studies, we have come to realize just how complex this exercise can be. Studies of homologous proteins come with a set of distinct caveats not present in other types of HX MS studies. As a tribute to the groundbreaking work of Brian Chait in the field of protein structure analysis by MS that was recognized with the 2015 ASMS Award for a Distinguished Contribution in Mass Spectrometry, we present a description of the caveats in homologous protein comparison experiments through our HX MS analyses of five closely related forms of the HIV-1 Nef protein.

Nef is a unique primate lentiviral accessory protein expressed in high concentrations shortly after infection and is essential for high viral loads in vivo [4–8]. Strains of HIV-1 isolated from individuals that remain AIDS-free for many years in the absence of antiretroviral drug treatment (long-term non-progressors or LTNPs) often display alterations to the *nef* gene [9–14], thereby implicating Nef as a critical virulence factor for the development of AIDS [15,16]. Targeted expression of Nef in transgenic mice induces a severe AIDS-like syndrome, identifying this protein as a key determinant of HIV pathogenicity [17]. Nef has

no known catalytic activity, and functions instead by interacting with a wide variety of host cell proteins to enhance viral infectivity and promote immune escape [4,18,19]. The ability of Nef to interact with diverse host-cell protein partners relates to its conformational plasticity.

Initial high-resolution structural data for Nef were obtained using deletion variants [20–23], as full-length Nef is prone to aggregation in the absence of partner proteins.. Residues 2–39 and 159–173 were removed for early solution NMR analyses of Nef [20,23] while deletion variants lacking residues 54–205 [21] or 58–206 [22] provided crystals suitable for structural determination. In these initial X-ray crystal structures, however, residues 54–69 and 149–178 remained disordered. The N-terminal region of HIV-1 Nef (residues 1–25 and 2–57) was also investigated by NMR [24,25] and found to be mostly disordered. All the known structural information for HIV-1 Nef was assembled into a useful model of the full-length protein by Geyer and Peterlin in 2001 [26]. In this model, the large internal loop (residues 157–174) was inserted with a probable conformation before energy-minimization.

Structural studies of HIV-1 Nef have also shed light on the mechanisms by which different regions and conformations of Nef regulate interactions with host cell effector proteins [20–22,27,28]. First-generation solution and crystal structures revealed how Nef binds to SH3 domains and forms homodimers, because these interactions required only the structured Nef core and not the N-terminal anchor region or internal flexible loop absent from these structures [20–22]. More recently, the crystal structure of a Nef:MHC-I peptide fusion protein in complex with the μ 1 subunit of the clathrin adaptor AP-1, an interaction essential for MHC-I downregulation and immune escape, revealed important details of the Nef N-terminal anchor domain conformation [27]. Along similar lines, the crystal structure of Nef bound to the AP-2 α - σ 2 subunit hemicomplex, an interaction responsible for CD4 downregulation, revealed a biologically important conformation for the Nef internal loop for the first time [28]. Finally, a recent structure of Nef in complex with the SH3-SH2 regulatory region of Hck revealed a dramatic reorganization of Nef dimer interface [29] relative to earlier structures of Nef bound to the SH3 domain alone. Taken together, these studies support the idea that the conformational plasticity of Nef is essential to its diverse functions.

Analysis of *nef* sequences from long-term non-progressors (LTNPs) revealed many scattered amino acid changes relative to alleles from laboratory and other virulent HIV-1 strains [26,30]. The conformational environment of these residues is unknown but many of the amino acid changes unique to LTNP Nef proteins fall outside of the structured core (Figure 1). We therefore hypothesized that the solution conformations of Nef from LTNPs may be different from those of laboratory strains that are commonly investigated (e.g., SF2, NL4-3) and that these conformational differences may help to explain the impaired Nef function associated with the LTNP phenotype.

To address these questions, we used HX MS to compare the conformation of full-length Nef proteins from multiple HIV-1 strains, including an LTNP-derived Nef protein (LTNP4) previously shown to be defective for Src-family kinase activation [31]. A particular advantage of using HX MS for these comparisons is that full-length Nef proteins, which are not amenable to NMR or crystallization due to poor solubility, can readily be probed by HX

MS because of the low protein concentration required for analysis ($< 25 \mu\text{M}$) [32]. We employed HX MS to compare the conformational properties of recombinant Nef proteins derived from the HIV-1 strains SF2, ELI, NL4-3, Consensus and LTNP4. All five Nef proteins share a high degree of sequence homology, especially in the structured core region (Figure 1A). However, the sequences are different enough to complicate HX MS analysis at the peptide level. For example, the five Nef proteins do not share identical pepsin digestion patterns, making the comparison of regional HX from protein to protein difficult. Despite these challenges, we show that these five Nef proteins are indeed different with respect to deuterium incorporation and SH3 binding characteristics. In the regions that could be compared across the proteins, there are significant differences in conformation for the portions of Nef that have been defined as being functionally relevant. More generally, our approach to the HX MS analyses of these Nef variants provides a framework for investigation of other protein relatives, families, and superfamilies.

MATERIALS and METHODS

Protein Expression and Purification

SF2, ELI, and LTNP4 Nef proteins were expressed in Sf-9 insect cells and purified as described previously [33]. Briefly, a hexahistidine tag was added to the N-terminus by PCR and the cDNA subcloned into the baculovirus transfer vector, pVL1392. A recombinant baculovirus was prepared by transfecting Sf-9 insect cells with the transfer vector and Baculogold DNA according to the manufacturer's protocol (BD-Pharmigen). Recombinant Nef proteins were purified using immobilized metal affinity chromatography. Purity and concentration were confirmed by SDS-PAGE, densitometry, Bradford assay and electrospray mass spectrometry. Consensus G2E and NL4-3 Nefs were expressed in *E. coli* and purified according to the protocol described in [31]. Human Hck SH3 domain was prepared by over expression in *E. coli* [strain BL21 (DE3) pLysS] as described in [34].

Deuterium Exchange Reactions

Nef proteins in equilibration buffer (20 mM Tris HCl pH 8.3, 100 mM NaCl, 3 mM DTT) were removed from -80°C storage and thawed on ice for approximately 10 minutes. The Nef proteins were then used as is or combined with the Hck SH3 domain to investigate exchange in the complex.

Deuterium labeling for the peptide analysis of the five Nef proteins alone was initiated with a 15-fold dilution of 1 μL Nef (50 pmoles) into D_2O labeling buffer. Labeling proceeded for specific amounts of time (10 seconds to 4 hours) and the reaction was quenched with the addition of an equal volume of quench buffer (150 mM potassium phosphate, pH 2.6). Samples were analyzed immediately following the quench step.

Protein: ligand HX MS experiments were performed at the global level for Consensus G2E or NL4-3 Nef (as ligand) bound to the human Hck SH3 domain (*SH3:Nef*), or at the peptide level for the human Hck SH3 domain (as ligand) bound to Consensus G2E, SF2, NL4-3, or LTNP4 (*Nef:SH3*). The *SH3:Nef* binding reaction was initiated with the mutual dilution of a stock solution of SH3 domain (120 μM) with Consensus G2E (280 μM , using K_D of 0.85

μM [35]) or NL4-3 (48 μM , using K_D of 1.4 μM [36]) in equilibration buffer. After preparation of the binding reaction, the protein mixtures were equilibrated together at 4 °C for one hour before the initiation of the labeling reactions. The *Nef:SH3* binding reaction was initiated with the mutual dilution of a stock solutions of SH3 domain (180 μM with Consensus and SF2 or 500 μM with NL4-3 and LTNP4) with Consensus Nef G2E (50 μM), SF2 Nef (50 μM , using K_D of 0.64 μM [35]), NL4-3 Nef (40 μM), or LTNP4 Nef (40 μM , using K_D of 0.25 μM [35]) in equilibration buffer. After preparation of the binding reactions, the protein mixtures were equilibrated together on ice for 30 minutes before the initiation of the labeling reactions.

Deuterium labeling for global analysis of the *SH3:Nef* binding reaction was initiated with a 15-fold dilution of 5 μL (60 pmol Hck SH3) of the binding reaction into D₂O labeling buffer (20 mM Tris pD 8.3, 100 mM NaCl, 3 mM DTT). Under labeling conditions and assuming the K_D values above 0.85 μM and 1.4 μM for Consensus G2E and NL4-3 bound to Hck SH3, respectively, Hck SH3 was 82% bound to Consensus Nef and 60% to NL4-3 (calculations performed as described in [37,38]). Labeling proceeded for specific amounts of time (10 seconds to 6 hours) and then the reaction was quenched with the addition of an equal volume of quench buffer (250 mM potassium phosphate pH 2.6). Samples were immediately frozen on dry ice and stored at -80 °C until mass spectral analysis.

Deuterium labeling for local analysis of the *Nef:SH3* binding reaction was initiated with a 15-fold dilution of 2 μL of the *Nef:SH3* binding equilibration mixture into D₂O labeling buffer. Under labeling conditions and assuming the listed K_D values, Nefs were bound to the SH3 domain at 84%, 87%, 91% and 98% for Consensus, SF2, NL4-3, and LTNP4, respectively. Labeling proceeded for specific amounts of time (10 seconds to 4 hours) and the labeling reaction was quenched with the addition of an equal volume of quench buffer (150 mM potassium phosphate pH 2.6). Samples were analyzed immediately following the quench step.

Analysis of Deuterium Incorporation

Global deuterium analysis—Labeled proteins were injected onto a self-packed POROS 20 R2 (Applied Biosystems) protein trap and desalted with 0.5 mL H₂O (0.05 % TFA) at a flow rate of 500 $\mu\text{L}/\text{min}$. The proteins were eluted into the mass spectrometer using a linear 15–75% acetonitrile gradient in 4 minutes at 50 $\mu\text{L}/\text{min}$ using a Shimadzu HPLC (LC-10ADvp). The HPLC was performed using protiated solvents which results in the removal of deuterium from the side chains and the amino/carboxy termini that exchange faster than backbone amide hydrogens [39]. All steps were performed with the protein trap, injector and associated tubing submerged in an ice bath. Mass spectral analyses were carried out with a Waters LCT-Premier^{XE} mass spectrometer with a standard electrospray source. The deuterium levels were not corrected for back-exchange [39] and are therefore reported as relative [32]. The average amount of back-exchange using the experimental setup described above was 12–15% based on analysis of highly deuterated protein standards. The relative deuterium incorporation for the SH3 domain was determined by subtracting the mass of the unlabeled protein from the mass at each labeling time. Each entire labeling time course experiment was performed in duplicate. After the elution of each sample from the LC

system into the mass spectrometer, an infusion of myoglobin (500 fmoles/ μ L) was used as a quasi-internal standard for calibration purposes. The error of determining the deuterium level at any time point averaged ± 0.4 Da. The isotope envelopes in bimodal patterns were fit with two Gaussian functions whose widths were estimated from the width of a single binomial isotopic envelope before and after the appearance of the bimodal pattern. The unfolding rate for SH3 domain spectra that presented evidence of a bimodal isotopic envelope was determined from the slope of pseudo-first-order kinetic plots of the decrease in the relative intensity of the lower mass envelope with time [34,40,41]. Slowdown factors were calculated as described in [34] (the unfolding $t_{1/2}$ for the SH3:Nef reaction divided by the $t_{1/2}$ for the SH3 domain alone).

Local deuterium analysis—Labeled samples prepared for the analysis of deuterium peptic peptides were analyzed using a custom Waters nanoACQUITY system [42]. Labeled samples were injected at a flow rate of 100 μ L/min into a 2.1 mm $\times$ 50 mm stainless steel column that was packed with pepsin immobilized on POROS-20AL beads (prepared as described in [43,44]) held at 15 $^{\circ}$ C. Under these conditions, the digestion time was approximately 30 seconds. Peptic peptides were trapped and desalted on a VanGuard Pre-Column trap (2.1 mm $\times$ 5 mm, ACQUITY UPLC BEH C18, 1.7 μ m) for 3 min. The trap was placed in-line with a Acquity UPLC BEH C18 1.7 μ m 1.0 $\times$ 100 mm column (Waters Corp.) and peptides were eluted into the mass spectrometer with a 8–40% gradient of acetonitrile over 6 min at a flow rate of 40 μ L/min. All mobile phases contained 0.1 % formic acid and the temperature of all components, excluding the pepsin column, was 0.1 $^{\circ}$ C. Mass spectral analyses were carried out on a Waters QToF Premier. Peptides produced from online pepsin digestion were identified using Waters ProteinLynx Global Server version 2.4 with Identity Informatics software. Deuteration levels were calculated using Waters DynamX software by subtracting the centroid of the isotopic distribution for peptide ions of an undeuterated sample from the centroid of the isotopic distribution of peptide ions from a labeled sample. The deuterium levels were not corrected for back-exchange [39] and are therefore reported as relative [32]. Each entire labeling time course experiment was performed in duplicate and the deuterium uptake values in uptake curves (as in Supplemental Figure S4) represent the average of the duplicate measurements.

RESULTS AND DISCUSSION

The proteins that were compared

HX MS has been used previously to study HIV-1 Nef. The conformation of both full-length HIV-1 Nef (strain SF2) and SIV Nef (mac239) were first studied in solution [45] and found to be consistent with the Geyer-Peterlin model of full-length Nef [26]. One significant advantage of HX MS is that the experiments are performed at protein concentrations of 25 μ M or less [45] where full-length Nef proteins are not prone to aggregation. Therefore, the dynamics of entire full-length protein – as opposed to deletion variants or shorter, more soluble constructs required for NMR – could be analyzed. Successful binding experiments utilizing HX MS include analysis of SF2 Nef [31] and Consensus G2E Nef binding to various Hck SH3 domains [46]. All prior Nef:SH3 binding HX MS experiments were done at the intact protein level.

Previous comparison of HIV-1 Nef alleles from eight LTNP4s identified a unique Nef protein (LTNP4) with impaired function in terms of Src-family kinase activation and association with the Hck SH3 domain [31]. These results led us to question the mechanism behind the defect in Nef LTNP4 function despite sequence similarity with the other patient and lab-adapted alleles. Amino acid sequence alignments of four well known laboratory strains of Nef with LTNP4 showed scattered differences across the backbone, with the sequence of ELI the most unique of the five proteins (Figure 1A). This observation is consistent with the fact that ELI Nef is derived from the HIV-1 D subtype, while the other lab alleles are B clade. Calculations of sequence conservation (Figure 1B) yielded sequence identities that ranged from 79.0 to 89.5%, with the lowest identity between NL4-3 and ELI and the highest between SF2 and Consensus G2E. The location of sequence differences do not map to obvious motifs involved in known Nef partner protein interactions, and thus did not explain the observed differences in SH3 domain binding or function [31]. Figure 1C shows an example for the amino acid differences between SF2 and LTNP4 Nef represented as red and grey balls on the Nef model structure. Many of the amino acid changes are conservative (e.g., Lys to Arg, Asp to Asn, Ile to Leu, grey balls) and not predicted to affect the overall conformation. The significant differences in sequence (e.g., Trp to Tyr, or Glu to Gly, red balls) which may change the conformation and therefore the function/binding were generally outside of the stable structured core of Nef and not in locations predicted to impact function.

HX MS on the intact protein level

We hypothesized that higher-order protein conformation may play a significant role in the function of Nef. This idea is based on the observation that seemingly subtle differences in the primary structure for the five forms of Nef did not map to interaction surfaces, and therefore may allosterically influence tertiary structure and conformational dynamics. Because the incorporation of deuterium into a protein backbone in solution is a function of both solvent accessibility and hydrogen bonding, any change to either of these parameters alters the level of deuteration [47]. Accordingly, HX MS was used in order to determine if subtle and scattered differences in primary structure translated into differences in tertiary structure, and therefore HX.

Initially, we chose to investigate the relative deuterium labeling of all five Nef proteins at the intact protein level, similar to the famous Katta and Chait studies of ubiquitin [1], to provide evidence for global differences in tertiary structure. Immediately we encountered problems in data interpretation. First, the maximum number of backbone amide positions that could be labeled in each Nef protein was different due to differences in the length of the proteins and in sequence differences that changed the number of proline residues [number of backbone amide hydrogens in each protein in parenthesis: Consensus-G2E (201), SF2 (213), ELI (207), NL4-3 (208), and LTNP4 (196)]. In an attempt to compensate for these differences, we converted the relative deuterium incorporation to relative percent deuterium level to compare exchange into the proteins at the global level (Figure 1D). This approach initially suggested the presence of global differences in deuterium incorporation. The largest difference in exchange (13%) was at the 1 minute time point between SF2 and ELI Nef. Interestingly, LTNP4 had a similar deuterium incorporation profile when compared with the other three laboratory strains. Overall, ELI Nef – which does not bind to Hck SH3 due to a

single point mutation in the RT-loop binding pocket [48] – incorporated less deuterium when compared to the other four Nefs. However, investigations of this nature – meaning HX MS studies of intact proteins with variable sequence – are fraught with problems. Differences in sequence change forward and back-exchange rates, and as we describe below, interpretation of the data as presented in Figure 1D is generally so confounded with problems that it becomes meaningless.

Difficulties in comparing HX MS when sequence is variable

The required considerations for correct interpretation of HX MS data from proteins with different sequences apply to both intact proteins and to the peptic peptides produced in many pepsin-level experiments. Because intact protein HX MS lacks spatial resolution, many studies involve digestion of deuterium-labeled proteins into peptides with the acid protease pepsin and then MS analysis of the deuterium level of each peptide as a function of time [39]. Major problems in data interpretation arise when the sequences of the proteins one wishes to compare are not identical and no correction has been made for back-exchange. To correct for back-exchange, a fully deuterated reference protein must be analyzed (recently reviewed in Ref. [49]) and a correction equation [39] applied. In the case of Nef, fully deuterated reference proteins could not be prepared and therefore all deuterium levels are reported as relative [32]. As a result, data interpretation is confounded by the following variables: (1) both forward- and back-exchange rates change with sequence, (2) extent and position of pepsin digestion can be different, (3) the LC retention time of peptides with different sequences can be different, and therefore the amount of time such peptides are exposed to quench conditions and undergo back-exchange can be variable, (4) direct comparison of deuterium levels for a given region of a protein, often done for relative deuteration comparisons, may not be possible if the identical peptides are not found. Point #1 applies to both intact protein and peptide HX MS while points 2–4 apply to peptide-level experiments. Of course, back-exchange correction would make the data analysis much more straightforward, as would single amino acid HX resolution; unfortunately, neither of these is possible for many proteins, including Nef. Our intent here is not to address how to analyze sequence variable proteoforms by applying back-exchange corrections or by obtaining single amino acid resolution, but rather to use this example of Nef proteins to discuss what one can do with relative deuterium incorporation data when sequences are variable. Detailed descriptions of methods for preparing totally deuterated controls and why those methods often fail for many proteins, as well as descriptions of the many issues related to the inability to perform single amino acid resolution studies in HX MS with ETD and related non-ergotic fragmentation process are topics for other future articles.

Hydrogen exchange rates for backbone amide hydrogens vary as a function of all levels of structure. Sequence affects the exchange rates because the amino acid sidechain on either side of a backbone amide hydrogen exerts influence over the kinetics of the exchange reaction; the extent of this effect can be calculated for any sequence [50,51]. Theoretical calculations of sequence-dependent exchange rates do not account for the influence imparted by secondary, tertiary and quaternary structure. To determine how much influence sequence may have over the exchange of the five forms of Nef, the theoretical backbone amide hydrogen exchange rates for all five Nef proteins were calculated according to [50]. Again,

these calculations assume the sequence adopts a completely unstructured form devoid of secondary, tertiary and quaternary structure. Both back exchange at quench conditions [pH 2.5, 0 °C – reported as back exchange half-life, or BE $t_{1/2}$] and forward exchange at the experimental conditions [pD 8.3, 21 °C – reported as k_{int}] were calculated. The full results plotted as a function of amino acid sequence are shown in Supplemental Figure S1. The average (across all backbone amide hydrogens) BE $t_{1/2}$ values for each protein were: Consensus-G2E: 159.08 min; SF2: 159.55 min; ELI: 170.74 min; NL4-3: 165.85 min; LTNP4: 164.63 min. The average forward-exchange (k_{int}) for each of the Nef proteins were: Consensus-G2E: 2649.89 min⁻¹; SF2: 2786.23 min⁻¹; ELI: 2858.25 min⁻¹; NL4-3: 2827.42 min⁻¹; LTNP4: 2848.73 min⁻¹. If we assume that all of the Nef proteins become equally deuterated before exposure to back-exchange conditions, ELI Nef would retain the most label followed by NL4-3, LTNP4, SF2 and Consensus G2E. Further, if we assume that secondary, tertiary and quaternary structure plays no role in the deuteration, Consensus-G2E and SF2 Nef would be more slowly deuterated than the other three Nef proteins. In reality, of course, secondary, tertiary and quaternary structure strongly influence the exchange rates and rankings based solely on forward- and back-exchange are therefore not observed (Figure 1D). These calculations are merely hypothetical examples to illustrate the point that with divergent sequences, one should not expect to observe the same level of deuterium incorporation by MS.

Digestion into peptides further complicates comparison of the five Nefs. The peptides that were followed during these experiments (encompassing 98, 88, 86, 90, and 82% of the amino acid sequences for Consensus G2E, SF2, ELI, NL4-3, and LTNP4 Nefs, respectively, not considering purification tags) are shown in the peptide maps in Supplemental Figure S2. This figure clearly shows that sequence variability gives rise to markedly different digestion patterns. Two specific examples of the theoretical calculations just described for intact proteins are shown for peptides in Figure 2; we will consider the effects of back-exchange first. It is a safe, but not always correct (see below) assumption that after digestion, peptides have no secondary, tertiary or quaternary structure to influence exchange. If this is true, then back-exchange during analysis therefore should be entirely a function of sequence and environmental conditions. The half-life for deuterium in backbone positions to revert to hydrogen for any of the Nef proteins spans three orders of magnitude between 1 and 1000 minutes (Supplemental Figure S1A). No peptide in this experiment underwent greater than 12 minutes exposed to back-exchange conditions (i.e., being in an all hydrogen environment from cold injection of the quenched reaction and online digestion, through UPLC separation and ultimately MS detection). Therefore, we have conservatively doubled this time and have used a BE $t_{1/2}$ of 30 minutes (represented as a purple dashed line in Figure 2A and Supplemental Figure S1A) as an artificial cutoff for amino acid residues that could “pose a problem”. “Posing a problem” means that in the analysis, any backbone amide hydrogen with a value that is below the 30 minute back-exchange line could lose enough label during analysis that one might expect to observe changes in the measured mass. An example of where this calculation could become important is a peptide that covers the PxxPxR region of Nef. This region is essential for SH3 domain binding [52] and is represented in all five Nef proteins by a 15 amino acid peptide with identical termini. However, only four out of the five proteins have identical sequences. NL4-3 has a threonine at position 6 instead of the

conserved arginine. Arguably, this single difference is significant in that what was once a large charged side chain has now been reduced to a shorter polar side chain. Additionally, there is also the ability of the arginine ϵ -NH to retain deuterium label under quench conditions and in the timescale of the LC MS analysis. Figure 2A, a larger version of this region from Figure S1, shows the calculation. Even when the sequence changes (R to T), altered back-exchange would not substantially change the relative deuterium incorporation graph. Back-exchange for the backbone amide hydrogens in this sequence ($^{71}\text{VG..MT}^{85}$, referred to hereafter as Peptide 1, see Supplemental Figure S3) is slow and well above the dotted purple line. Therefore, we have chosen to designate this region as acceptable for comparison despite the sequence differences. The MS results for deuteration of this peptide can likely be compared and the minor difference observed in the uptake graph (Supplemental Figure S3, peptide 1) can be considered as meaningful.

As illustrated with the preceding example, theoretical calculation of back-exchange becomes relevant when comparing a specific peptide region that does not have the same amino acid sequence or length. Back-exchange during analysis, in addition to being affected by sequence, can be affected by the actual conditions of the analysis. A recent study [53] illustrated this point with a series of deuterated peptides of varying lengths derived from a single protein as a means to increase special resolution. Rates of back-exchange for the same sequence but of varying length can be different. Peptides of varying lengths and hydrophobicity theoretically have differing retention times during the LC separation step and subsequently varying percentages of back-exchange based on the combined effects of the sequence differences and the amount of time exposed to quench conditions. There may also be secondary structure even in quench conditions and sequence may affect secondary structure and the theoretical amount of back-exchange that is to be expected. Therefore, even peptides that are identical in much of the sequence but differ by a few amino acids in length should be treated as suspect. Figure S3 shows various examples of different scenarios in the comparison of peptides across all five of the Nef proteins. Peptides 3 and 4 in Figure S3 differ by only a few amino acids, but would a comparison of relative percent deuteration be valid given the difference in length? Another example is at the N-terminus. A peptide region in the N-terminus (far left of Figure S3) certainly cannot be compared due to changes in peptide length and sequence. For this region, the relative deuterium uptake graph and any figure derived from it (e.g., Figure S3, top, and Figure 3) cannot be interpreted with certainty because differences in deuterium labeling could be due to differences in back-exchange rather than differences in conformation. As already mentioned above, correction for loss of deuterium during analysis (if possible) and not relying merely on measurements of the relative amount of deuterium incorporation is one solution to such comparison dilemmas.

We also want to mention that the intrinsic rate of backbone amide hydrogen forward-exchange during the labeling step (k_{int}) is another important consideration. The k_{int} is a fundamental property of the primary structure of a protein and is independent of secondary, tertiary and quaternary structure. Differences in the intrinsic rate can also be viewed as an initial level of comparison amongst homologous proteins as the primary structure not only dictates k_{int} , but also it drives/creates both secondary and tertiary structure. For HX MS investigations of protein variants measured under conditions of equivalent pH, ionic strength, and temperature, would the k_{int} for the backbone amide hydrogens within

structurally homologous regions alter what one would measure by MS for such a region? For the Nef variants, the intrinsic rates of exchange for all backbone amides were calculated at experimental conditions of labeling (pD 8.3 and 21 °C), as shown in Supplemental Figure S1B. As in back-exchange, those peptides that have similar sequences will have similar values for k_{int} , as illustrated with the top panel of Figure 2B [note that the scenario in Figure 2B is what may happen in HX MS of point mutants, again emphasizing that even small changes to sequence must be considered in data interpretation]. For those regions where there is more sequence variability, such as the N-terminus of these five Nef proteins, comparison of HX data in this region across all of the variants is more difficult. Assuming no secondary, tertiary or quaternary structure, the calculations for a region from $^7\text{KR}..^{\text{VR}}17$ in Nef (Figure 2B, bottom panel) shows that the rate of exchange for a single backbone amide hydrogen can vary by two orders of magnitude. Imagine then a scenario in which the k_{int} was different between two protein variants in a structurally homologous region (e.g. a solvent-exposed loop). Differences in the amount of deuterium incorporation could be driven solely by these sequence differences in regions where secondary, tertiary and quaternary structure do not override (by slowing) the rate of exchange calculated from sequence alone.

What can be compared?

Considering both the differences in the peptides produced during online digestion and the differences in the rates of intrinsic forward- and back-exchange over the five Nef sequences, we were only able to do head-to-head comparisons for just under 30% of the Nef sequence across all five proteins. Not surprisingly, this mostly included regions of the protein core where the sequence was less variable (see Figure 1C as an example). Given that some comparisons of data are not valid, another complication is how to faithfully indicate differences in deuterium incorporation for the five proteins while visually representing the deuterium incorporation data in the most effective manner for valid cross-variant comparison.

We have chosen to present the peptide data as relative percent deuterated (calculated from the measured deuterium level divided by the deuterium level if all possible backbone amide hydrogen positions were deuterated). However, the comparison of deuterium exchange data for peptides of varying lengths and sequences using a relative percent deuterated scale generally is not valid. A detailed section at the beginning of the Supplemental Material has been included to illustrate pitfalls in comparisons of relative percent deuterated data. We describe two instances where the direct comparison of different peptides is not valid and a third that illustrates that the comparison does have merit in certain instances (e.g., a comparison of percent deuterium incorporation can be a valid exercise for two peptides of identical sequence with slightly different lengths at either the N- or C-termini because the total possible exchange sites and amino acid lengths of the peptides are similar). In illustrating peptide-level data for the Nef proteins, we have chosen to employ both vertical heat maps (Figure 3) and the more traditional deuterium incorporation plots (Figures 4, S3, and S4).

It is possible and informative to compare changes to these five Nef proteins when interacting with a protein partner, the Hck SH3 domain. Comparisons of the effects of binding are not

influenced by all of the caveats discussed above because the peptides in free and bound forms remain the same. The pattern and extent of pepsin digestion is much less affected by the presence of other proteins during digestion. Therefore, relative deuterium measurements in binding experiments of various related proteins can provide reliable information about how sequence variation affects interactions even when direct comparisons of the exchange of the related proteins suffers from multiple data interpretation complications.

Comparison of Nef proteins at the peptide level

The results of the Nef HX digestion experiments, both free and bound to the Hck SH3 domain, are visually summarized in Figures 3 and 4 and all deuterium incorporation plots can be found in Figure S4A–E. Figure 3 makes it easy to quickly see how the Nef proteins become deuterated (panel A) and where there are/are not differences upon binding (when comparing panel A and B, as described below). As discussed above, a direct comparison across all peptides derived from each of the Nef proteins – that is comparing data between the proteins horizontally across Figure 3 – is not a valid exercise. There are regions that meet the criteria outlined above for valid comparison of deuterium uptake, including the Nef core and four peptide regions (labeled 1–4 on Figure 3 and in Figure S3)

Peptide regions 1, 2, and 3 have nearly the same deuterium incorporation profiles across all five Nef proteins. Region 1 covers the PxxPxR motif responsible for binding to Src-family kinase SH3 domains and there were only slight differences in deuterium exchange for the free Nef proteins in this region. Regions 2 and 3 are both within the folded core of each Nef variant, and the exchange data were largely similar here; this is not unexpected as these regions have nearly identical amino acid sequences. There was no sequence coverage for ELI Nef in the peptide 2 region, so a complete comparison is not possible. Peptide region 3, which encompasses β -sheets 1 and 2, shows nearly identical deuterium incorporation for the three B-clade laboratory strains, while both ELI (clade D) and the primary LTNP4 Nef proteins are distinct. However, this region of the Nef core has not been ascribed any specific function.

Peptides in area 4 overlap almost completely with a short Nef α -helix (helix 4) that is induced when in complex with the AP-2 endocytic adaptor protein [28]. This interaction is essential for Nef to remove CD4 receptors from the surface of infected cells through the AP-2 endosomal trafficking pathway. The location of the Nef area 4 peptide is modeled on the crystal structure of the Nef:AP-2 complex in Supplemental Figure S5A. In this region, the rate of deuterium incorporation is indistinguishable for Consensus G2E, SF2, and LTNP4, while NL4-3 may be slightly reduced. However, deuteration of this region is significantly delayed for ELI in comparison to the other forms, raising the question of whether this difference is reflected in Nef function related to CD4 downregulation. To address this possibility, we compared the extent of CD4 downregulation by each of these Nef proteins in the SupT1 T-cell line by flow cytometry. As shown in Figure S5B, ELI Nef was substantially impaired for CD4 downregulation, with only 14% of the cell population demonstrating reduced cell-surface CD4 levels compared with 54 to 86% for the other Nef variants tested. Differences in deuterium uptake in this region may reflect altered dynamics that may in turn impact interaction with AP-2 and subsequent CD4 downregulation.

Comparison of Nef variants in the SH3-bound state

As alluded to above, differences in HX observed upon partner protein binding should not be affected by alterations to intrinsic exchange rates as a result of sequence changes because data are compared for the same protein in unbound vs. bound states. To illustrate this point, we explored the HX MS changes resulting from SH3 domain binding to each of our Nef variants. Nef modulates signaling of pathways linked to Hck and other Src-family kinases by binding to their SH3 domains, leading to constitutive kinase activation. Previous studies have shown that the SF2, ELI, and LTNP4 variants of Nef have different binding affinities for the SH3 domain of Hck [31]. In that study, the change in deuteration of Hck SH3 when bound to a ligand was compared to deuteration of unbound Hck SH3 to calculate a slowdown factor (see Methods and Materials). A larger slowdown factor means tighter binding. SF2 Nef bound tightly to SH3 (large slowdown factor), LTNP4 Nef bound weakly and ELI Nef did not bind at all, and these binding interactions correlated well with Hck activation by each of these Nef proteins [31]. In the current study, we included two additional forms of Nef: Consensus G2E and NL4-3, neither of which had previously been studied by HX MS (Figure S6). HX MS data suggest that Consensus-G2E Nef binds as tightly to SH3 as SF2, while NL4-3 binds less well and in a manner that is similar to LTNP4 Nef. ELI Nef does not bind at all, consistent with previous work [48]. While the binding activities of these five Nef alleles towards the Hck SH3 domain vary, HX data from the Nef SH3-binding region (peptide 1 in Figure S3) are nearly identical, consistent with the high degree of sequence conservation in this area. Therefore, other factors besides the presence of the PxxPxR sequence must substantially contribute to the interaction between Hck SH3 and Nef. Indeed, early structures of the Nef:SH3 complex revealed a major role for the three-dimensional fold of the Nef in complex formation [21]. By extension, intrinsic protein dynamics are also likely to have a major impact as well as other protein-protein interactions essential to Nef function.

We next examined the impact of SH3 binding on each Nef protein at the peptide level by digesting the deuterium labeled SH3-bound Nef proteins and comparing those data to the unliganded proteins. ELI Nef does not bind to Hck SH3 [31] and served as a negative control. In addition to looking for changes in the PxxPxR region, we were interested to see if there were any other changes to Nef conformation in the presence of the SH3 domain. Figure 3B shows the relative percent deuterium for Nef peptides derived from the SH3-bound form, which can be compared directly to the relative percent deuterium level of the peptides derived from the apo form in Figure 3A. In this Figure, comparisons of the relative deuterium level in the same protein between panel A and B (vertical) is a valid comparison while comparison across alleles (horizontal) is subject to the caveats described above. That said, visual inspection reveals that deuterium uptake across most of the Nef proteins remains unchanged as a function of SH3 binding. However, two regions show significant differences across the Nef proteins, and these are described in detail in the next section.

Two peptide regions with similar sequences could be reliably compared across the Nef proteins as a function of SH3 binding (peptides 1 and 2; Figure 3–4). Peptide 1, which encompasses the PxxPxR motif, showed an initial decrease in deuterium incorporation in the presence of Hck SH3 across all four Nef proteins (Figure 4A). The initial protection from

exchange at the early time points is evident for all of the Nef proteins tested (compare gray lines to colored lines). Interestingly, there is also a difference in the timing of the dynamic behavior of the backbone in this region for each Nef variant in the presence of the SH3 domain. This is apparent when the differences between the deuterium incorporation curves (SH3-bound minus free) are plotted on the same axes (Figure 4A, panel v). LTNP4 becomes labeled more quickly relative to SF2 and NL4-3, while Consensus G2E is intermediate. Peptide 2, which encompasses the C-terminal half of Nef α -helix 2 and faces the Nef:SH3 interface, exhibits a decrease in deuterium incorporation for the longer exchange time points. The difference between Nef bound and free for this peptide (Figure 4B, panel v) indicates that differences in deuteration upon binding are the result of a decrease in backbone dynamics in this region when Nef is bound to SH3. The change comes earliest for Consensus G2E Nef.

The major implication of these results for HIV-1 Nef is that previously unrecognized conformational differences may play a role in reduced Nef function. Despite minor sequence variation between the Nef alleles, the analysis of the HX peptide data suggests that differences in deuterium incorporation are the result of proteins with slightly different solution conformations and altered binding capacity. It is tempting to speculate as to how the conformational differences seen between these Nef proteins translate into functional differences. In some important functional regions, there were clear differences in deuterium exchange and therefore in conformation/dynamics. Overall, HX reveals that LTNP4 appears to be a more protected, intrinsically rigid molecule. Whether individual alterations in the amino acid sequence can explain the substantial protection afforded to LTNP4, or whether it is the sum of all of these changes, remains unknown. Perhaps Nef proteins from LTNPs are more conformationally restricted in general and hence less well adapted to form the many conformations required for full biological activity. A large-scale conformational characterization of a broad spectrum of Nef alleles by HX MS, including all major clades and multiple LTNPs, will help to clarify the role of conformational changes in impaired LTNP Nef function.

CONCLUSIONS

Comparing HX MS at the peptide level for related proteins of variable sequence is far removed from the much more straightforward measurement of HX MS in a single protein as was described by Brian Chait so many years ago. Other considerations and the magnitude of their influence will likely not be known until much more HX MS data for protein families is acquired and analyzed. This example of five Nef proteins is on the small side of such comparison experiments that are currently in progress. The caveats that must be considered for comparison experiments, especially when making relative deuterium measurements, are many and must all be considered. As was shown here, certain types of experiments – namely binding experiments – can provide a great deal of information about how related proteins behave.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

LTNP	long-term non-progressor
HX	hydrogen exchange
MS	mass spectrometry

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Figure 1.

Properties of the Nef alleles in this study. (A.) Sequence alignment of Consensus (Cons), SF2, ELI, NL4-3, and LTNP4 alleles of HIV Nef. Residues that differ in this alignment are highlighted with yellow. Elements of secondary structure are indicated on the top of the alignment: green lines for α -helices, and blue lines for β -sheets. The PxxP motif is indicated with a red line. Regions of the Nef sequence that have been studied using NMR [20,23,24] or X-ray crystallography [21,22,27,28] are indicated under the sequence. (B.) Sequence conservation between each of the Nefs, as calculated by ClustalW. (C.) Tertiary locations of amino acid differences between SF2 and LTNP4 Nef, as an example comparison. Comparison was made on the Nef structural model by Geyer et al. [26]. The amino acid changes are indicated near each colored ball with red balls indicating dramatic substitutions and grey balls indicating conservative substitutions. The structural core [22] has been indicated with a molecular surface. (D.) Intact protein HX MS of the five Nef proteins. Error bars are not indicated in this figure (see Materials and Methods).

Figure 2.

Theoretical amide hydrogen exchange rates for selected peptides/regions of all five Nef proteins. The complete calculations for the entire proteins are found in Supplemental Figure S1. Consensus G2E: solid orange circles; SF2: hollow green squares; ELI: hollow blue triangles; NL4-3 grey x marks; LTNP4: hollow red circles. (A.) The half-life of back-exchange (BE $t_{1/2}$, in minutes) at pH 2.5 and 0 °C in the PxxPxR region of Nef. The purple dashed line marks BE $t_{1/2}$ value of 30 minutes. Proline residues, which do not have backbone amide hydrogens, are indicated at the top of the BE $t_{1/2}$ axis but do not have a numeric value. (B.) Rates of forward exchange (k_{int} , in min^{-1}) at pD 8.3 and 21.0 °C for peptides covering the PxxPxR region (top panel) and an N-terminal region (bottom panel) of

Nef. Proline residues, which do not have backbone amide hydrogens, are indicated at the bottom of the k_{int} axis but do not have a numeric value.

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Figure 3.

Vertical heat maps of the deuterium incorporation into peptides of HIV variants. The peptic peptides for Nef free in solution (A.) and bound to the HckSH3 domain (B.) are shown as colored bars extending from the N-terminus (top of each panel) to the C-terminus (bottom of each panel). The length of each peptide is proportional to the vertical height and overlapping peptides are excluded from this view for clarity. The location of every 50th amino acid is shown with horizontal lines. The deuterium level at each exchange-time (from left to right: 5s, 10 s, 1 min, 10 min, 1 h, and 4 h, as shown at top of ConsG2E panel) is shown in relative percentage, according to the color code shown. Peptide regions 1–4 as discussed in the text are indicated as vertical bars to the right of the ConsG2E panels. More details about the peptides used to create this figure are shown in Supplemental Table S1.

Figure 4. Regions of NeF that showed a difference in peptide HX upon Hck SH3 binding. (A.) Deuterium incorporation plots for the peptide spanning the PxxPxR motif and identified as peptide 1 on Figure 3. The location of this peptide is colored red in the structural model at top left above a sequence alignment. (B.) Deuterium incorporation plots for the peptide identified as peptide 2 in Figure 3. The location of this peptide is colored blue in the structural model at top left above a sequence alignment. For both panels A and B, results are shown for Consensus G2E (subpanel *i* in both A and B), SF2 (*ii*) NL4-3 (*iii*), and LTNP4 (*iv*). The calculated relative differences (Hck SH3 bound NeF – NeF alone) for each of the NeFs are shown in subpanel *v*. Error bars represent the minimum and maximum data achieved from duplicate measurements. All deuterium incorporation plots are in Supplemental Figure S4.