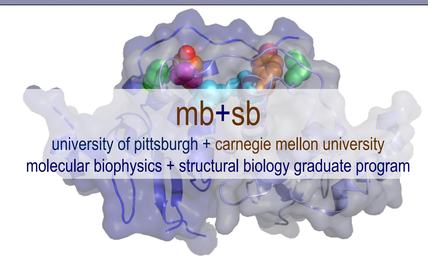
Activation of the Tec-family Kinases Itk and Btk by HIV-1 and SIV Nef Proteins *in vitro*

Manish Aryal[1,2], Kindra Whitlatch[1,2], John J. Alvarado[2], Thomas E. Smithgall[2]

Molecular Biophysics and Structural Biology Graduate Program[1] , University of Pittsburgh and Carnegie Mellon University

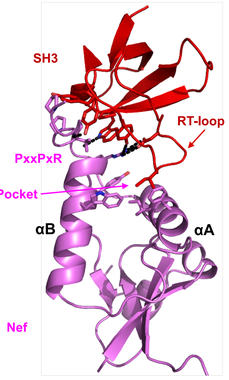
Department of Microbiology and Molecular Genetics[2], University of Pittsburgh School of Medicine



Background

Nef, Src and Tec Family kinases

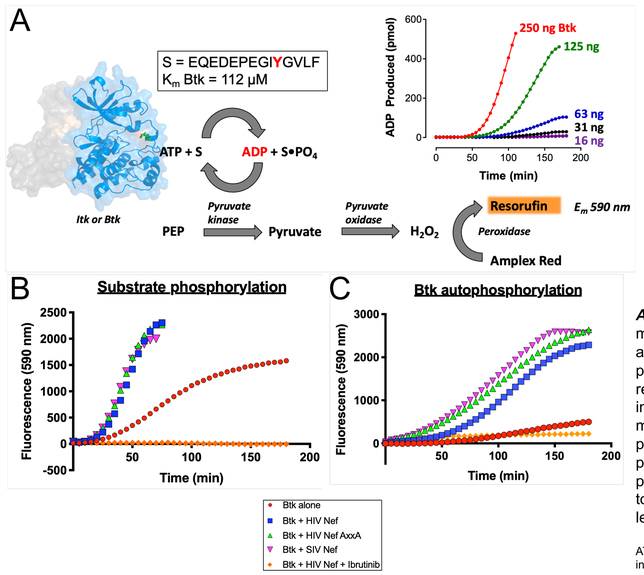
- Nef is an HIV-1 accessory protein required for immune evasion, high-titer viral replication and disease progression[1]
- Nef binds and activates both Src (Hck, Lyn, c-Src)[2] and Tec family kinases (Itk, Btk, Bmx)[3] and induces constitutive kinase activation
- Src and Tec family kinases exhibit a similar arrangement of regulatory SH3 and SH2 domains and auto-inhibited structures[4]
- Nef dimerization is required for Hck activation, CD4 and MHC-I down-regulation and virus replication[5]
- Inhibitors of Nef-induced Src- and Tec-family kinase activation block Nef-dependent HIV-1 replication *in vitro*
- Selective inhibition of Itk and siRNA knockdown blocks multiple steps in the HIV-1 life cycle[6]

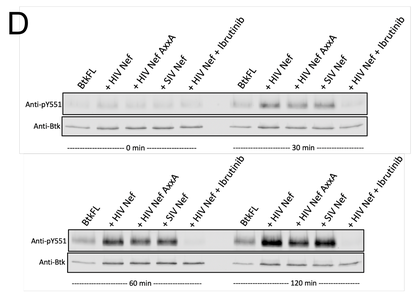


Nef_{core}:HckSH3

PDB ID:4U5W

HIV-1 and SIV Nef strongly activate Btk

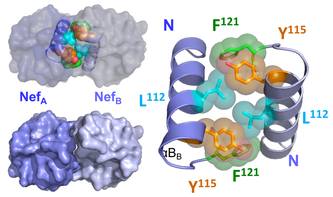




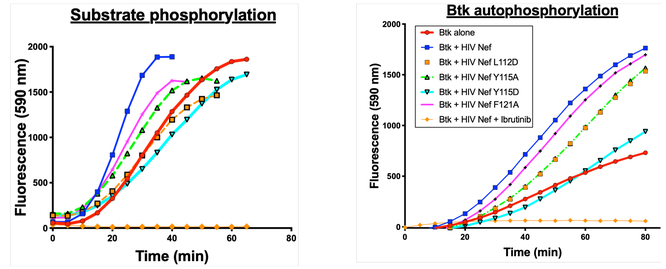
A*)** *Schematic of ADP Quest Assay (DiscoverX)*. This assay measures the amount of ADP produced as a result of kinase activity. ADP is detected in a coupled enzyme reaction that produces H_2O_2, which is used to generate the fluorphore, resorufin. Full-length recombinant Btk was added to the assay in increasing amounts to estimate the amount that yields the half maximal rate for use in reactions with Nef. Peptide substrate phosphorylation (B***) and Btk autophosphorylation (***C***) in the presence of indicated Nef proteins. ***D***) Immunoblots of Btk in presence of the indicated Nef proteins. Anti-BTK pY551 was used to probe Btk activation loop phosphorylation, while Btk protein levels were detected with an anti-His antibody.

Assay conditions (final volume 50 μL): 125 ng Btk (300 ng for immunoblots), 95 μM ATP, 112 μM substrate peptide, and 1 μM Btk-specific inhibitor ibrutinib (where indicated), pH 7.4. Nef proteins were added in 10-fold molar excess. ADP Quest assays were run in quadruplicate and the average values are shown.

Nef homodimer formation is required for Btk activation *in vitro*

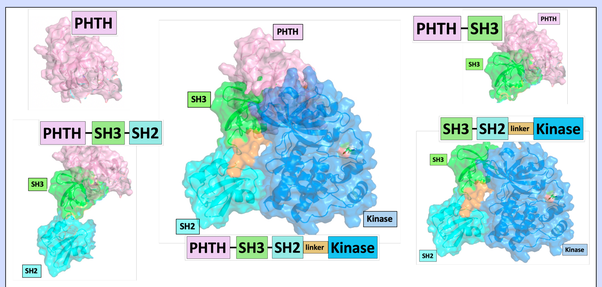


Crystal structure of an HIV-1 Nef homodimer in complex with the Fyn SH3 domain (*not shown*). The Nef dimerization interface is formed by the αB-helices and involves the side chains of Leu112, Tyr115, and Phe121 as shown. Previous work has shown that mutations at these positions perturb Nef homodimerization in a cell-based fluorescence complementation assay[5].



The effect of HIV-1 Nef dimer interface mutants on full-length Btk activation was assessed using the ADP Quest Assay. The rates of peptide substrate phosphorylation and Btk autophosphorylation were measured with Btk alone and in the presence of full-length wild-type Nef as well as the L112D, Y115A, and Y115D mutants.

Recombinant Btk and Itk proteins used in this study

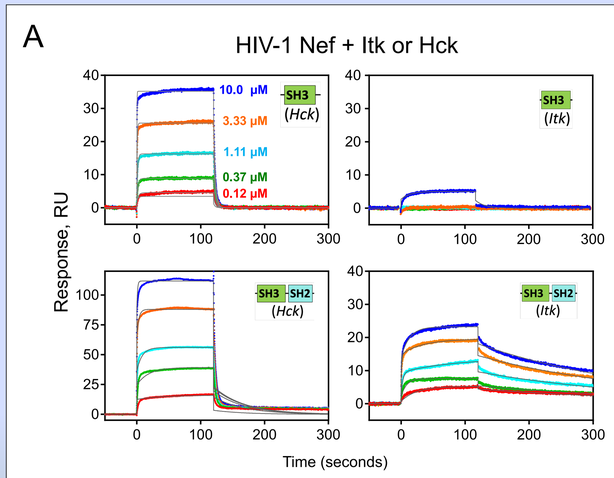


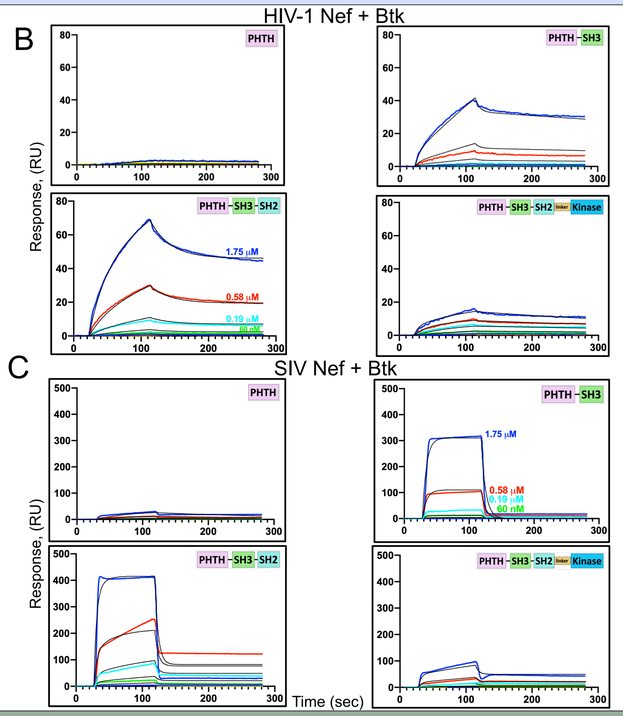
Recombinant full-length (PHTH-SH3-SH2-Kinase) and near-full-length (SH3-SH2-Kinase) Btk and Itk proteins were expressed in Sf9 insect cells with N-terminal FLAG or 12-Histidine tags, respectively. Kinase proteins were purified to homogeneity using anti-FLAG antibody or immobilized metal affinity chromatography (IMAC), followed by size-exclusion chromatography (SEC). Regulatory domain constructs (PHTH, PHTH-SH3, PHTH-SH3-SH2) were expressed in *E. coli* as either a N-terminal GB1 or C-terminal 6-His fusion proteins followed by purification using IMAC and SEC. Diagrams of Btk were rendered using PyMol and PDB coordinates 4XI2.

References

1. Foster, J. L. & Garcia, J. V. HIV-1 Nef: At the crossroads. *Retrovirology* (2008). doi:10.1186/1742-4690-5-84
2. Alvarado, J. J., Tarafdar, S., Yeh, J. I. & Smithgall, T. E. Interaction with the Src homology (SH3-SH2) region of the Src-family kinase Hck structures the HIV-1 Nef dimer for kinase activation and effector recruitment. *J. Biol. Chem.* **289,** 28539–53 (2014).
3. Tarafdar, S., Poe, J. A. & Smithgall, T. E. The accessory factor Nef links HIV-1 to Tec/Btk kinases in an Src homology 3 domain-dependent manner. *J. Biol. Chem.* **289,** 15718–28 (2014).
4. Wang, Q. *et al.* Autoinhibition of Bruton's tyrosine kinase (Btk) and activation by soluble inositol hexakisphosphate. *Elife* **4,** e06074 (2015).
5. Poe, J. A. & Smithgall, T. E. HIV-1 Nef Dimerization Is Required for Nef-Mediated Receptor Downregulation and Viral Replication. *J. Mol. Biol.* **394,** 329–342 (2009).
6. Readinger, J. A. *et al.* Selective targeting of ITK blocks multiple steps of HIV replication. *Proc. Natl. Acad. Sci.* **105,** 6684–6689 (2008).

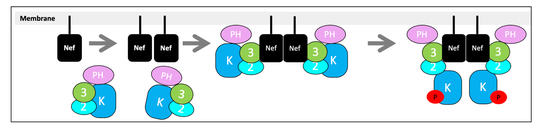
Analysis of Btk and Itk regulatory domain interaction with HIV-1 and SIV Nef by SPR





A*)** Surface plasmon resonance (SPR) analysis of HIV-1 Nef association with Hck or Itk regulatory domains. This analysis was carried with Nef covalently immobilized on a CM5 SPR chip as ligand and the Hck or Itk proteins flowed over the chip surface as analyte. Hck SH3 and SH3-SH2 domains stably bind HIV-1 Nef. Unlike Hck SH3 domain, the Itk SH3 demonstrates poor binding to Nef. However, the Itk SH3-SH2 dual domain protein shows stable binding to Nef. SPR sensorgrams showing immobilized HIV-1 Nef (B***) and SIV Nef (***C***) binding to the indicated Btk regulatory domains. Analytes were injected at a maximum concentration of 1.75 μM. Btk PHTH-SH3-SH2 exhibits the maximum binding to both HIV-1 and SIV Nef, suggesting that multiple domains are required for stable interaction.

Conclusions



Model of HIV-1 Nef induced activation of Btk (macrophages) and Itk (CD4 T cells). Dimerization of Nef on membrane recruits downregulated kinases in a way that promotes trans-phosphorylation between two Btk or Itk proteins thus leading to constitutive kinase activation, a process essential for viral replication in PBMCs[6].

- Full-length Itk and Btk are activated by both HIV-1 and SIV Nef proteins *in vitro*.
- Nef does not activate the Itk or Btk Src-like core kinases, suggesting a key role for the PH domain *(data not shown)*.
- Unlike the Src-family kinase Hck, both Itk and Btk are activated by a PxxP mutant of Nef, suggesting a distinct activation mechanism independent of the SH3 domain.
- The isolated Itk SH3 domain does not interact with HIV-1 Nef, consistent with SH3-independent activation.
- Nef residues involved in homodimerization are required for Btk activation, supporting a mechanism of kinase domain *trans*-phosphorylation.
- Ongoing studies are exploring the structural basis of Nef-mediated Btk activation by co-crystallization of Btk/Nef complexes.