HHS Public Access

Author manuscript

Nat Chem Biol. Author manuscript; available in PMC 2012 March 26.

Published in final edited form as:

Nat Chem Biol. ; 7(9): 585–587. doi:10.1038/nchembio.612.

An Orthosteric Inhibitor of the Ras-Sos Interaction

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Abstract

Mimics of α-helices on protein surfaces have emerged as powerful reagents for antagonizing protein-protein interactions, which are difficult to target with small molecules. Herein we describe the design of a cell-permeable synthetic α-helix based on the guanine nucleotide exchange factor Sos that interferes with Ras-Sos interaction and downregulates Ras signaling in response to receptor tyrosine kinase activation.

Aberrant receptor tyrosine kinase (RTK) signaling is a major underlying cause of various developmental disorders and hyperproliferative diseases.[1] A primary transduction mechanism by which RTK signals are propagated involves the ligand-dependent activation of the small guanine nucleotide binding protein Ras (Fig. 1a).[2] The rate-limiting step in this activation process is the conversion of Ras-GDP to Ras-GTP through an exchange reaction that is catalyzed by the Ras specific guanine nucleotide exchange factor Sos.[3] Accordingly, the design of inhibitors that target Sos-mediated Ras activation should constitute an effective strategy for experimental and therapeutic intervention.[4]

Structural and biochemical analyses of Ras-Sos interactions have demonstrated the involvement of multiple inter- and intra-molecular interactions that act in concert to destabilize the nucleotide-bound state of Ras.[3] A key element of this catalytic process is the disruption of direct and water-mediated interactions between Ras and guanine nucleotide by the insertion of a helical hairpin from Sos into the switch regions of Ras (Fig. 1b). Since the αH helix is the only portion of the hairpin that makes direct contact with Ras, we reasoned that α-helical mimics of αH could interfere with Ras-Sos interaction. Computational[5] and experimental mutational[6] analyses identified F929 and N944 as residues that contribute

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A.P., K.K.Y., P.S.A. and D. B.-S. designed experiments, analyzed data and wrote the paper, and A.P. and K.K.Y. performed experiments.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Supplementary information is available online at http://www.nature.com/naturechemicalbiology/.

most strongly to the binding of αH to Ras (Supplementary Results, Supplementary Table 2). Thus, we initiated the design of stabilized helices that mimic the full length (929–944) Sos αH helix. To this end we utilized the hydrogen bond surrogate (HBS) approach to design stabilized α-helical peptides (Fig. 1c).[7] The HBS strategy affords preorganized α-helices in which the N-terminal main chain hydrogen bond between the C=O of the i^{th} amino acid residue and the NH of the i+4th amino acid residue is replaced with a carbon-carbon bond. HBS helices have been previously shown to target their chosen protein receptors with high affinity and specificity.[8,9]

Synthetic mimics of the wild-type Sos αH (929–944) were only partially soluble in aqueous buffers at 25 μM and higher concentrations. We therefore optimized the native peptide sequence by incorporating charged residues at non-interfacial positions to enhance solubility. During this iterative design process, we also simultaneously examined the sequences for their helical content by circular dichroism spectroscopy and their potential to inhibit Ras/Sos association in an in vitro nucleotide exchange assay.[10] Replacement of non-essential hydrophobic residues and substitution of β-branched residues, which have low helix-forming propensities,[11] with suitable residues that favor the helical conformation resulted in an optimized sequence FEGIYRLELLKAEEAN. Detailed discussion of our peptide design strategy along with properties of various sequences is included as Supplementary Results. HBS helices were synthesized as previously described (Supplementary Fig. 1).[12] The key step in the synthesis of these compounds consists of a ring-closing metathesis reaction between two appropriately placed alkene groups on the resin bound peptide. One of the olefin coupling partners is installed by appending 4-pentenoic acid to the *N*-terminal amino acid residue, while the other olefin is incorporated as an *N*-allyl group at the *i*+4 position.

The optimized HBS helix, **HBS 3**, was judged to be 56% helical in 10% trifluoroethanol in phosphate buffered saline (Supplementary Fig. 3). When assayed in a cell-free nucleotide exchange reaction, this compound displayed a potent inhibitory effect as compared to a control analog **HBS 7** in which three residues that are important for interaction (F929, E942, N944) were substituted for alanine (Fig. 1d and Supplementary Fig. 4).[6] Circular dichroism spectroscopy confirmed that alanine substitutions do not affect the α-helicity of **HBS 7**. We find that **HBS 3** alone does not trigger the exchange of mantGDP from Ras (Supplementary Fig. 4); this observation is consistent with structural and biochemical studies indicating the requirement for multiple contact points between Sos and Ras to affect guanine nucleotide exchange.[3,6,13] Dissociation constants for binding of **HBS 3** and **HBS 7** to Ras were determined by fluorescence polarization assay using fluorescein-labeled peptides with GDP bound Ras and under conditions known to promote nucleotide dissociation (Supplementary Methods). **HBS 3** targets nucleotide-free Ras with a K_D of 28 ± 4.8 μM and GDP-bound Ras with a K_D of 158 ± 16 μM (Supplementary Fig. 5). By comparison, the reported K_D value for the interaction of nucleotide-bound Ras with the catalytic domain of Sos is 14.5 μM.[10] Whether this difference reflects the restricted number of contacts formed between **HBS 3** and Ras relative to the catalytic domain of Sos or a distinct mode of binding remains to be established. **HBS 7** binds Ras with a ten-fold lower affinity (273 ± 8.5 μM), indicating the specificity of **HBS 3**–Ras interactions (Supplementary Fig. 5a).

To further characterize the interaction of the peptide with Ras, we performed ^{1}H-^{15}N HSQC NMR titration experiments with **HBS 3** and uniformly ^{15}N-labeled recombinant Ras.[14] Addition of **HBS 3** to 150 μM Ras in 1:3 and 1:5, Ras:**HBS 3** ratios provided a concentration-dependent shift in resonances of several Ras residues (Supplementary Fig. 6a). Specifically, addition of **HBS 3** led to shifts in resonances of residues corresponding to the shallow cleft into which the native Sos αH helix binds (Fig. 2a and Supplementary Fig. 6b-c). This cleft consists of switch I (residues 25–40) and switch II (residues 56–75) regions, and spans the nucleotide-binding pocket, supporting our prediction that **HBS 3** can act as a direct mimic of αH. Shifts in peaks corresponding to residues that flank switch I and switch II regions were also observed (Supplementary Fig. 6b).

To assess the capacity of the designed αH helix mimetic to antagonize the RTK-mediated Ras activation, we first monitored the cellular uptake of fluorescein-conjugate derivatives by fluorescence microscopy. Cells incubated with **HBS 3** or **HBS 7** displayed an intense intracellular fluorescence signal in comparison to cells treated with fluorescein alone (Supplementary Fig. 7a-b), validating the suitability of **HBS 3** and its derivatives for cell-based assays. It is noteworthy that unconstrained analog **3** has low cell permeability consistent with earlier reports documenting the inferior biological activity of unconstrained peptides relative to their constrained counterparts.[9,15] The cellular uptake of fluorescein-labeled **HBS 3** is significantly reduced at 4 °C as compared to 37 °C (Supplementary Fig. 7b), suggesting that the stabilized helix does not passively diffuse into the cell but rather is internalized through an energy-dependent mechanism. The identity of this mechanism remains to be established.

We next sought to determine whether the treatment of cells with **HBS 3** can modulate Sos-mediated Ras activation in response to ligand-dependent receptor stimulation. Serum-starved HeLa cells were treated for 12 hours with **HBS 3** or control peptides (**3** and **HBS 7**) and then stimulated with EGF. Cellular levels of Ras-GTP were monitored by the Raf1 Ras-binding domain (RBD) pull-down assay.[16] Consistent with previous reports EGF stimulation resulted in a twenty-fold increase in Ras activation. The extent of Ras activation was significantly reduced in the presence of **HBS 3** (Fig. 2b **and** Supplementary Fig. 8). In contrast, incubation of cells with control peptides did not appreciably affect the levels of Ras activation. Notably, at the concentration and incubation times tested, the peptides did not alter the level or phosphorylation status of EGFR upon EGF treatment (Supplementary Fig. 9), suggesting that **HBS 3** targets Ras activation downstream of EGFR.

To further ascertain that the observed cellular effects of **HBS 3** are mediated through the direct inhibition of Ras-Sos interaction and not through receptor targeting or modulation of interactions upstream of Ras/Sos complex formation, we utilized a SosCat-CAAX construct in which the catalytic domain of Sos is tagged to the CAAX motif of Ras. This construct has been previously shown to anchor Sos to the membrane and activate Ras independent of growth factor stimulation.[17] Treatment with **HBS 3** resulted in an approximately 5-fold reduction in Ras-GTP levels when compared to untreated serum-starved HeLa cells expressing SosCat-CAAX (Fig. 2c **and** Supplementary Fig. 8). Taken together with the lack of apparent effect of **HBS 3** on EGFR activation, this result provides a strong indication that

the inhibition of Sos-mediated Ras activation by **HBS 3** is a consequence of its interference with Sos-Ras interaction.

The activation of Ras leads to stimulation of various signal transduction pathways.[18] To determine whether the inhibitory effect of **HBS 3** on Ras can modulate downstream signaling events, we focused on the activation of the ERK cascade, a well-documented Ras effector pathway implicated in cell proliferation and differentiation.[19] Serum-starved HeLa cells were pretreated with the indicated peptides and then stimulated with EGF for various time intervals. We monitored the resulting variations in the levels of ERK phosphorylation by blotting the cell lysates with phospho-ERK-specific antibodies. Significantly, **HBS 3** reduced the extent and duration of ERK activation post EGF addition (Fig. 2 d-e and Supplementary Fig. 8).[19] In comparison to **HBS 3**, the unconstrained counterpart and the specificity control have no apparent effect on ERK activation. The capacity of **HBS 3** to induce inhibition of ERK activation suggests that it could in principle be equally effective in compromising other Ras effector pathways downstream of the Ras-Sos interaction.

Binding of Sos to Ras triggers a substantial conformational reorganization involving switch I and switch II regions of Ras.[3] This organization exposes the nucleotide binding pocket and facilitates exchange. We find that a rationally designed stabilized α-helix that mimics a key element from Ras-binding domain of Sos can inhibit Sos-induced activation of Ras and regulate MAPK signaling in cultured cells. Significantly, the unconstrained peptide derivative remains inactive highlighting the remarkable potential of stabilized helices and helix mimetics in inhibiting previously untargeted protein-protein interactions.[15,20–22]

Overstimulation of Ras signaling is an inevitable consequence of constitutively active RTKs and accounts for a plethora of molecular changes that underlie various cancers and other diseases.[23,24] The present study identifies a strategy for intercepting at a key control point the pathway linking multiple RTKs to Ras signaling – Sos-mediated Ras activation. Adaptation of this strategy for therapeutic intervention may therefore offer a unique opportunity to pan-target a wide spectrum of disorders associated with aberrant RTK function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the US National Institutes of Health (GM073943 to P.S.A. and GM078266 and an ARRA stimulus supplement (GM078266S1) to D.B.-S.). A.P. thanks NYU for the Margaret and Herman Sokol Fellowship. NMR data were collected at the New York Structural Biology Center. NYSBC is a STAR center supported by the New York State Office of Science, Technology, and Academic Research. We thank Chin Lin (NYU), Aswin Natarajan (CUNY City College) and Ronnie Ghosh (CUNY City College) for help with the NMR studies.

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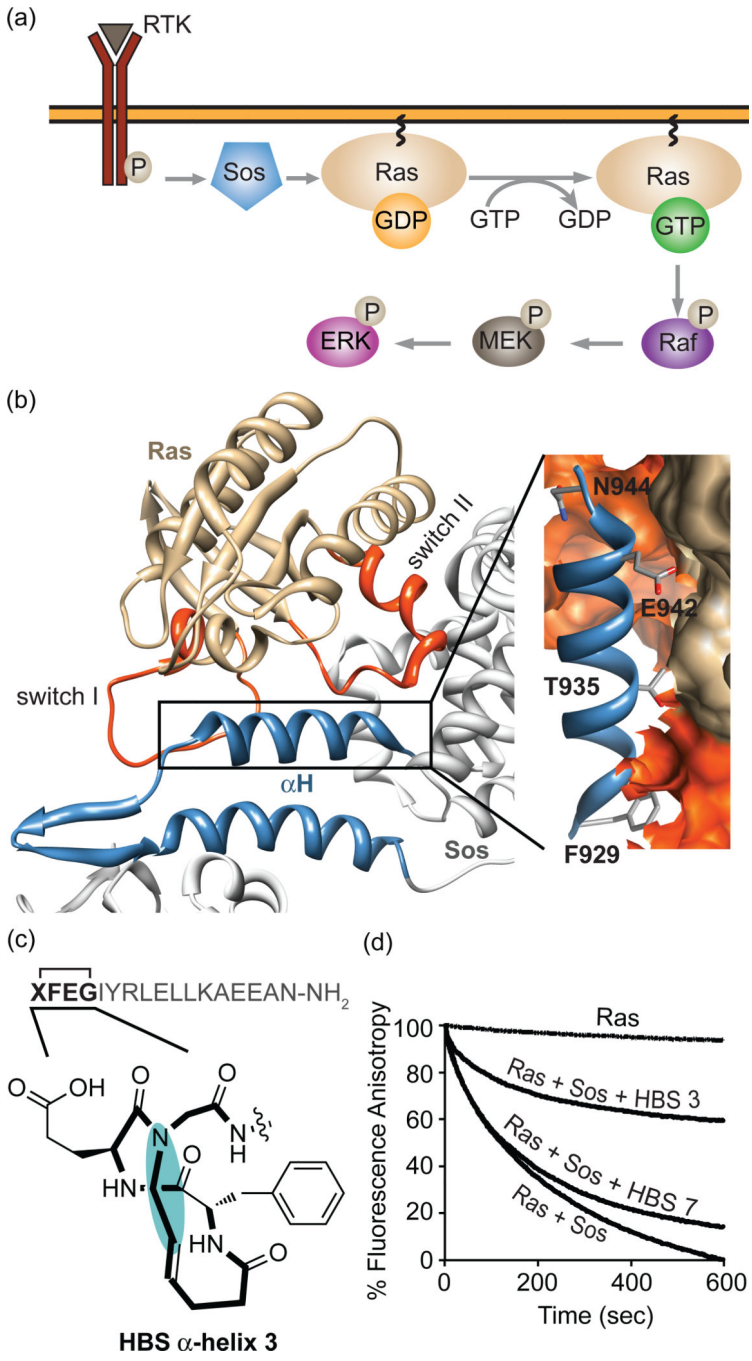

Figure 1. The Ras-Sos interface and rational design of synthetic inhibitors

(a) Schematic depiction of the major transduction steps in the RTK-Sos-Ras-ERK pathway. Binding of growth factor to RTK leads to its phosphorylation triggering recruitment of Sos to the plasma membrane. Membrane-localized Sos activates Ras by facilitating exchange of GDP for GTP. Activated Ras stimulates the ERK-MAP kinase cascade through the sequential phosphorylation of Raf, MEK and ERK.

(b) Ribbon diagram showing the region within the Ras-Sos interface containing the Sos helical hairpin (blue) (PDB code 1NVW). The hairpin inserts into the flexible switch regions

of Ras (orange). The αH motif makes direct contacts with the switch regions of Ras with residues F929, T935, E942 and N944 of Sos contributing significantly to complex formation (inset).

(c) The hydrogen bond surrogate (HBS) helices feature a covalent bond in place of the intramolecular hydrogen bond between the *i* and *i+4* residues (blue). Sequence of the optimized Sos αH mimetic, **HBS 3**, is shown.

(d) Rates of nucleotide exchange from Ras in the presence or absence of Sos and αH mimetics. **HBS 3** significantly suppresses nucleotide exchange as compared to the negative control, **HBS 7**.

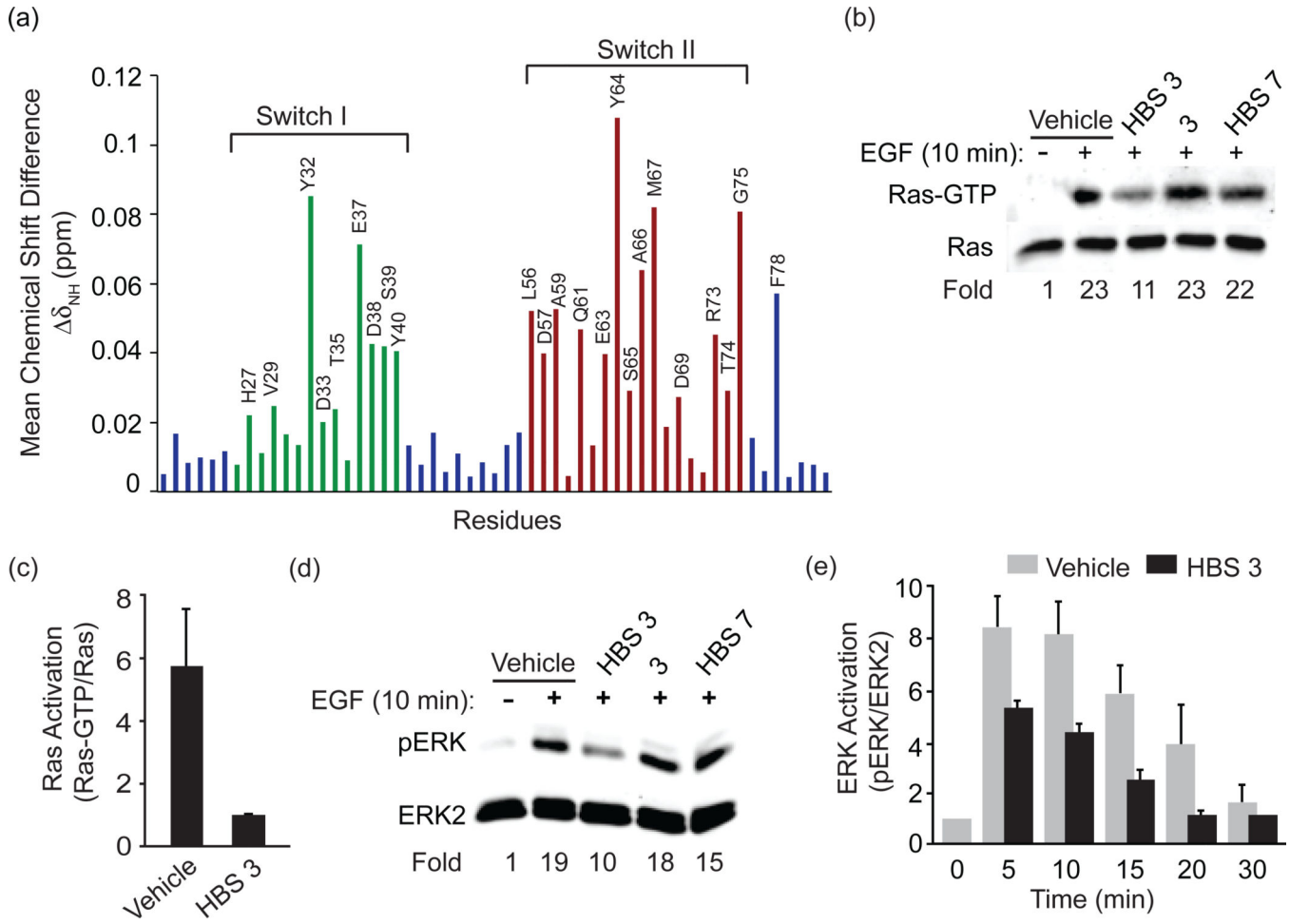

Figure 2. HBS 3 inhibits the Ras-ERK pathway

(a) Mean chemical shift difference (ΔdNH) plot depicting changes in residues spanning the switch regions upon the addition of increasing amounts of **HBS 3**. Switch I region is shown in green, Switch II in red and the flanking non-switch regions in blue.

(b) **HBS 3** attenuates EGF-induced Ras activation while the specificity control **HBS 7** and the unconstrained analog **3** are ineffective. Representative immunoblot is shown; fold activation refers to the levels of active Ras relative to the level measured in the absence of EGF.

(c) **HBS 3** downregulates Ras activation by interfering with the Ras-Sos complex independent of growth factor stimulation. Results are the mean ± SD of three independent experiments and are presented as the level of active Ras in untreated cells relative to the level measured in treated cells.

(d) **HBS 3** specifically suppresses EGF-induced ERK activation as compared to **HBS 7** and the unconstrained analog **3**. Representative immunoblot is shown; fold activation refers to the levels of pERK measured relative to the levels in the absence of EGF.

(e) **HBS 3** reduces the intensity and the duration of EGF-induced ERK activation. Results are the mean ± SD of three independent experiments and are presented as the levels of pERK relative to the levels measured in the absence of EGF.