Free Radical Biology & Medicine 52 (2012) 444–451

Contents lists available at SciVerse ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Peptide inhibitors of the Keap1–Nrf2 protein–protein interaction

Rowena Hancock [a], Hélène C. Bertrand [a], Tadayuki Tsujita [b], Shama Naz [a], Ayman El-Bakry [a], Jitnueng Laoruchupong [a], John D. Hayes [b], Geoff Wells [a,*]

[a] Department of Pharmaceutical and Biological Chemistry, School of Pharmacy, London WC1N 1AX, UK
[b] Biomedical Research Institute, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, Scotland, UK

ARTICLE INFO

Article history:
Received 23 August 2011
Revised 26 October 2011
Accepted 26 October 2011
Available online 3 November 2011

Keywords:
Fluorescence polarization
Cancer chemoprevention
Compound screening
Nrf2
Keap1
Free radicals

ABSTRACT

Disruption of the interaction between the ubiquitination facilitator protein Keap1 and the cap'n'collar basic-region leucine-zipper transcription factor Nrf2 is a potential strategy to enhance expression of antioxidant and free radical detoxification gene products regulated by Nrf2. Agents that disrupt this protein–protein interaction may be useful pharmacological probes and future cancer-chemopreventive agents. We describe the structure–activity relationships for a series of peptides based upon regions of the Nrf2 Neh2 domain, of varying length and sequence, that interact with the Keap1 Kelch domain and disrupt the interaction with Nrf2. We have also investigated sequestosome-1 (p62) and prothymosin-α sequences that have been reported to interact with Keap1. To achieve this we have developed a high-throughput fluorescence polarization (FP) assay to screen inhibitors. In addition to screening synthetic peptides, we have used a phage display library approach to identify putative peptide ligands with non-native sequence motifs. Candidate peptides from the phage display library screening protocol were evaluated in the FP assay to quantify their binding activity. Hybrid peptides based upon the Nrf2 "ETGE" motif and the sequestosome-1 "Keap1-interaction region" have superior binding activity compared to either native peptide alone.

The possible role of dietary constituents in cancer prevention has focused interest on foodstuffs with biological activity and the components that may modify the risk of developing neoplastic disease. Of particular interest are compounds that activate nuclear factor-erythroid 2-related factor 2 (Nrf2) and thereby up-regulate a battery of about 100 antioxidant response element-driven genes that protect against carcinogenesis and degenerative conditions [1–3]. Nrf2 induces the expression of a number of genes involved in protecting cells against free radicals, electrophiles, and oxidative stress, in particular the glutathione synthesis enzymes (e.g., γ-glutamate–cysteine ligase), proteins involved in protein-thiol homeostasis (e.g., thioredoxin-1, thioredoxin reductase-1, sulfiredoxin-1), and phase I and II metabolic enzymes (e.g., NAD(P)H:quinone oxidoreductase-1, glutathione *S*-transferases). Many of the natural product activators of Nrf2 antagonize its interaction with Kelch-like ECH-associated protein-1 (Keap1) [4], a substrate adaptor for the Cul3–Rbx1 E3 ubiquitin ligase that represses Nrf2 by targeting it for proteasomal degradation [5–7]. In particular, many compounds that induce Nrf2-target genes are thiol-reactive and prevent Cul3–Rbx1 ubiquitination of Nrf2 by alkylating or oxidizing key cysteine residues in Keap1 [6,8–10]. An alternative strategy to using soft electrophiles to perturb the interaction between Keap1 and Nrf2 is to interfere directly with the protein–protein interface. Such an approach has the potential advantages of reversibility and specificity, due to a non-covalent binding mechanism, and reduced off-target effects.

The interaction between Nrf2 and Keap1 proteins has been characterized at the molecular level. Data from deletion and mutagenesis experiments indicate that two separate regions within the N-terminal Neh2 domain of Nrf2, the "DLG" motif (residues 24–31) and the "ETGE" motif (residues 78–82), interact with the Kelch-repeat domain of the Keap1 protein (the C-terminal region of Keap1) [11], at a site called the "Kelch binding pocket" on the bottom surface of Keap1 [12,13]. Both the DLG and the ETGE motifs are required for Keap1-directed ubiquitination of Nrf2 by Cul3–Rbx1 [14]. NMR studies suggest that the Neh2 domain of Nrf2 is relatively unstructured in solution, but becomes organized upon association with the Keap1 protein [11]. Mutagenesis, crystallography, and isothermal calorimetry studies have indicated that the DLG motif has relatively low affinity for Keap1 ($K_D \approx 1000$ nmol/L) whereas the ETGE motif has a higher affinity ($K_D \approx 5.3$ nmol/L) [15]. Both the DLG and the ETGE sequences form β-turn-like structures and interact with Keap1 primarily via salt bridges between acidic Asp and Glu residues in the peptide and Arg residues 380, 415, and 483 in the Keap1 Kelch domain [15]. The ETGE motif forms more of these electrostatic interactions than the low-affinity DLG sequence. We have used these structural biology data to inform the design of an assay to identify compounds that inhibit the protein–protein interaction (PPI) between Nrf2 and Keap1.

In addition to its ability to bind Nrf2, Keap1 can also interact with other intracellular proteins such as prothymosin-α [16,17] and

* Corresponding author. Fax: +44 20 7753 5964.
E-mail address: geoff.wells@pharmacy.ac.uk (G. Wells).

0891-5849/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2011.10.486

sequestosome-1 (also called p62) [18–21]. In the case of sequestosome-1, the region that binds to Keap1 has been called the "Keap1-interaction region"; mutagenesis studies have shown it closely resembles the ETGE motif in Nrf2 [18,21]. However, unlike Nrf2, prothymosin-α and sequestosome-1 appear to interact with Keap1 through only a single binding site. It has been reported previously that ubiquitination of Nrf2 by Cul3–Rbx1 requires the transcription factor to be tethered upon Keap1 through two binding sites [14], and so it seems unlikely that prothymosin-α and sequestosome-1 are ubiquitinated by Cul3–Rbx1. An interesting possibility is that prothymosin-α and sequestosome-1 compete with Nrf2 for binding to Keap1 and thus augment the expression of genes regulated by Nrf2.

In this paper, we describe the development of a homogeneous and high-throughput assay based upon the principle of fluorescence polarization [22] to screen and quantify the binding of ligands to the Kelch binding pocket of Keap1. The Keap1 binding assay has been applied to an array of synthetic peptides based upon DLG- and ETGE-containing sequences in Nrf2, peptides derived from prothymosin-α and sequestosome-1, and the hit sequences obtained from screening a phage display peptide library [23] to identify inhibitors of the PPI. We have developed detailed structure–activity relationships based upon these data that provide information on relative binding affinities and can be applied to future small-molecule and peptidomimetic drug design approaches. The data relating to sequestosome-1 modifications suggest that phosphorylation of a Ser residue in its binding motif could regulate the interaction between sequestosome-1 and Keap1.

Materials and methods

Materials

All chemicals were obtained from commercial suppliers and used without further purification. Dimethylformamide, dichloromethane, trifluoroacetic acid (TFA), and methanol were purchased from Aldrich. Ninhydrin, piperidine, triisopropylsilane (TIS), diisopropylethylamine, and fluorescein isothiocyanate (FITC) were purchased from Fisher. All α-amino acids, resins, and coupling reagents were obtained from NovaBiochem. The carboxyfluorescein-labeled peptide FAM-LDEETGEFLP (**5**) was purchased from Cambridge Research Biochemicals. The peptides **9** and **12–15** were obtained from the Cancer Research UK peptide synthesis facility. Phosphate-buffered saline (PBS; pH 7.4), dithiothreitol (DTT), and EDTA were from Sigma. Surfactant P-20 was provided by Biacore Life Sciences. Tris (10 mmol/L, pH 7.4) and Hepes (10 mmol/L, pH 7.5) buffers were prepared according to standard protocols.

Peptide synthesis

The remaining peptides were synthesized using manual Fmoc solid-phase peptide synthesis using the appropriate preloaded 2-chlorotrityl or Wang resins. The peptides were cleaved and deprotected for 2 h using 95% TFA, 2.5% water, and 2.5% TIS. The crude, ether-precipitated peptides were purified by reversed-phase HPLC and lyophilized. The molecular masses were confirmed by electrospray ionization mass spectroscopy and structure was confirmed using ^{1}H NMR.

Plasmids

The expression vector pET15bhKelch, which encodes the Kelch-repeat domain of human Keap1 (residues 321–609) fused at its N-terminus to a hexahistidine tag, has been described previously [24]. The expression vector pMal-mKeap1$^{\Delta 1-307}$, encoding the Kelch-repeat domain of mouse Keap1 (residues 308–624) fused at its N-terminus to maltose-binding protein (MBP), has been described elsewhere [14]; in this study, this is called pMal-mKelch. Site-directed mutagenesis was used to create pMal-mKelch^{G364C} and pMal-mKelch^{G430C} mutants. An expression vector, designated pET15bmNeh2, encoding the Neh2 domain of mouse Nrf2 (residues 1–96) attached at its N-terminus to a hexahistidine tag, was prepared by PCR amplification of a mNrf2 cDNA [25], with the following primers: 5′-CCACAGCGTCCGCCCTC**CATATG**ATGGACTTGGAGTTGCCACCG-3′ (forward) and 5′-CAACCTGGGAGTAGCTGGCG**CTCGAG***CTA*GGTGTCTGTCTGGATATGCTG-3′ (reverse) (the nucleotides that are in boldface represent the NdeI and XhoI restriction sites, and those in italics represent those mutated to create a termination codon). After digestion with NdeI/XhoI, the PCR product was ligated into pET15b (Novagen) that had been similarly digested.

Protein expression and purification

Human Kelch protein was heterologously expressed in *Escherichia coli* from pET15bhKelch as a His-tagged polypeptide and purified in three steps: first, by nickel-chelation chromatography on a HiTrap Chelating HP column (GE Healthcare Life Sciences); second, by anion-exchange FPLC on a Mono Q column (GE Healthcare Life Sciences); and third, by gel-filtration liquid chromatography on a Superdex 75 pg column. The MBP mouse Kelch protein and MBP mouse Kelch^{G364C} and MBP mouse Kelch^{G430C} mutant proteins were expressed in *E. coli* from pMal-mKelch and the fusion proteins were purified using columns of amylose resin (New England Biolabs). The His-tagged mouse Neh2 protein was expressed in *E. coli* from pET15bmNeh2 and purified by nickel-chelation chromatography, followed by anion-exchange Mono Q chromatography and, last, size-exclusion chromatography on a Superdex 75 pg gel-filtration column.

Phage display

To identify peptides that bound the Kelch-repeat domain of Keap1, a commercially available phage display (Ph.D.) peptide library that expresses random linear dodecapeptides (New England BioLabs) was screened. Briefly, 60-mm plastic dishes (Iwaki) were first coated with MBP mouse Kelch (MBP-mKelch) fusion protein (100 μg/ml in 0.1 M $NaHCO_3$, pH 8.6) by overnight incubation at 4 °C. After being blocked for a further 2 h at room temperature with 5 mg/ml bovine serum albumin (BSA) in 100 mmol/L $NaHCO_3$ buffer, pH 8.6, containing 0.02% sodium azide, a portion of the phage library (1.5×10^{11} phage in 1 ml of TBST; TBS containing 0.1% v/v Tween 20) was added to the dish where it could bind MBP-mKelch for 1 h at room temperature. Thereafter, the dish was washed with TBST to remove unbound phage. The remaining bound phage particles were then eluted with 200 mmol/L glycine–HCl buffer, pH 2.2, containing 1.0 mg/ml BSA, before being neutralized and amplified in ER2738 *E. coli* cells. After three further rounds of "panning" with MBP-mKelch protein (in which the peptides that bound specifically to MBP-mKelch were selected), the remaining phage were subjected to a final panning round, again using MBP-mKelch fusion as the bait, but were selectively eluted in a step-wise manner by a buffer providing different stringencies: first, to recover phage with weak affinity, wells were washed with TBS containing 1 mmol/L DLG 9-mer peptide (i.e., H_2N-LDIDLGVFL-OH; **12**); second, to recover phage with moderate affinity, wells were washed with TBS containing 1 mmol/L ETGE 9-mer peptide (i.e., H_2N-LDEETGEFL-OH; **9**); third, to recover phage with high affinity, wells were washed with 200 mmol/L glycine–HCl buffer, pH 2.2, containing 1.0 mg/ml BSA. The phage eluted by the ETGE peptide and those eluted by the low-pH buffer were studied separately. Within each of these two pools, the phage that bound the MBP part of the fusion protein bait, rather than the Kelch domain, were removed by adsorption to MBP alone and discarded. Thereafter, the unbounded phage in the two pools were screened on the basis of their ability to bind to mutant MBP-mKelch^{G364C} and MBP-mKelch^{G430C} proteins; those that bound the mutant proteins were