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Peptides identify multiple hotspots within the ligand binding domain of the TNF receptor 2

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Abstract

Background: Hotspots are defined as the minimal functional domains involved in protein:protein interactions and sufficient to induce a biological response.

Results: Here we describe the use of complex and high diversity phage display libraries to isolate peptides (called Hotspot Ligands or HSPLs) which sub-divide the ligand binding domain of the tumor necrosis factor receptor 2 (TNFR2; p75) into multiple hotspots. We have shown that these libraries could generate HSPLs which not only subdivide hotspots on protein and non-protein targets but act as agonists or antagonists. Using this approach, we generated peptides which were specific for human TNFR2, could be competed by the natural ligands, TNFα and TNFβ and induced an unexpected biological response in a TNFR2-specific manner.

Conclusions: To our knowledge, this is the first report describing the dissection of the TNFR2 into biologically active hotspots with the concomitant identification of a novel and unexpected biological activity.

Background

Hotspots have been defined as the minimal functional protein:protein interaction domains through which biological activity can be modulated [1–3]. A novel strategy called Phenogenix® has been developed as a means for studying these interactions. To enable Phenogenix®, we use large and diverse phage display libraries consisting of randomized 20 mer and 40 mer amino acid peptides with > 10^{11} independent clones. Because of their ability to modulate protein: protein interactions, the resulting peptides are called Hotspot Ligands (HSPLs). Using this approach, we have successfully identified peptide agonists and antagonists for a number of biologically important molecules including growth hormone receptor, insulin receptor and the insulin-like growth factor receptor [2,3].

In this report, we describe the use of Phenogenix® to identify the critical protein:protein interactions underlying the TNF/TNFR axis. TNFα and TNFβ have been extensively studied and are involved in immune and pro-inflammatory responses, playing an important role in host defenses against infection and other disease states [4–7]. The biological effects of TNFα and TNFβ are mediated through the two membrane associated receptors, TNFR1 (p55) and TNFR2 (p75), that are expressed on the target cells [8,9]. The postulated pathogenic roles for TNF include sepsis and other bacterial and viral pathologies [10–12], certain

Table 1: Receptor-Specific Hotspot Ligands Are Identified By Biopanning TNFR2.

KcC7	GLQRWCRWAESACGDSGPLR
KcD11	AT**W**Q**EY**FR**RGG**SCSLAESCG
KcF6	CWR**W**I**EY**WS**RGG**QVGRRAGA
Motif	**W**x**EY**xx**RGG**
KcF12	SVVAWTGALRPSWVELRCLREVSDSGKMHPAWLDYCGELS

The sequences shown are the deduced amino acid sequences of the human TNFR2-specific phage clones that were selective for TNFR2, but not TNFR1. The cysteine residues are underlined and highlighted in grey. A potential motif, **WxEYxxRGG**, found in peptides KcF6 and KcD11 are indicated as bolded one letter amino acid codes.

cancers [13], metastasis [14] and chronic autoimmune disorders such as rheumatoid arthritis [15,16], multiple sclerosis [17] and Crohn's disease [18]. It is worth noting that TNFα mutants have been identified that selectively bind TNFR1. One such mutant induced less systemic toxicity yet showed no compromise in its anti-tumor activity in nude mice [13]. These observations indicated that finding modulators specific for each of the TNF receptors is indeed possible.

While TNFα binds equally well to both receptors, the majority of biologic responses attributed to TNF are mediated via TNFR1 although there is evidence that activation of TNFR2 is responsible for some adverse effects of TNFα [19–24]. Thus, the differential regulation of these two receptors may require drugs that specifically target either TNFR1 or TNFR2 allowing therapeutic efficacy without the expected toxicity profile. One obvious possibility then is to search for Surrogates that selectively bind and regulate the individual receptors. To date several antagonistic protein reagents have been isolated for treating diseases with an underlying TNF etiology. These include neutralizing monoclonal antibodies and soluble forms of TNFR1 and TNFR2 [25–27]. However, both antibodies and extracellular receptors are large proteins and not amenable to oral administration. They also carry the unwanted risk of stimulating an auto-immune response during chronic use.

By panning our peptide libraries against the TNFR2, we isolated four HSPL peptides binding specifically to human TNFR2 that allow the subdivision of the receptor into a minimum of at least three independent hotspots involved in its interaction with TNFα. In a biological model for TNFα activation of TNFR2, three of the four peptides induced cytotoxic response in the target cell line suggesting that at least one of these domains induced a unique TNFR2-specific biological response. Overall, these data show the utility of using the HSPL peptides for subdividing protein:protein interacting domains and for defining unique novel biological activities not observed through the natural ligand/receptor interaction.

Results

Human TNFR2 was panned with random 20 mer and 40 mer phage display peptide libraries of high diversity [2,3,28]. After four rounds of panning, four phage clones were isolated with specificity for TNFR2. The peptides were initially divided into two groups: a 40 mer designated KcF12 and three 20 mers designated KcC7, KcD11 and KcF6 (Table 1). From the amino acid sequences, KcC7, KcD11 and KcF12 were found to have a pair of cysteine residues suggesting the possibility of intra-chain disulfide bonding. In addition, a putative motif, WxEYxxRGG, was found for peptides KcF6 and KcD11 (Table 1). It was interesting that this motif was related to a sequence WxExxxxGG found at the C-terminal (amino acids 190–198) of TNF-α. No appreciable sequence homology was seen between either of the natural ligands, TNFα or TNFβ, and KcF12 although the spacing of cysteine loop was close to that seen in the cysteine rich domains of TNFR2 [29]. At present, it is not known whether these structural elements play a role in binding of the peptides to TNFR2 and it may be that the peptides mimic rather than recapitulate the contact domains of the natural ligands. ELISA analysis showed that all four phage clones bound specifically to human TNFR2 and did not react with either human or murine TNFR1 or murine TNFR2 (data not shown).

Competition ELISAs were performed using TNFα and TNFβ vs. the four phage clones at their ED_{50} values (Figure 1a,1b,1c,1d). More TNFα and TNFβ were required to compete KcF12 than the other phage clones suggesting that certain intrinsic properties of KcF12 (such as its length and/or amino acid content) may contribute to a higher relative affinity (Table 2). In addition, the synthetic forms of KcC7, KcD11, KcF6 and the recombinant KcF12 (rKcF12) were able to compete the binding of TNFα and TNFβ, as well as KcF6 and KcF12 phage clones in the micromolar range (unpublished data). Interestingly, the three 20 mer HSPLs but not KcF12 were competed by an anti-TNFR2 monoclonal antibody, mAb226, (Figure 2) suggesting that KCF12 binds to a hotspot on TNFR2 distinct from the other 3 peptides. A summary of the phage/peptide competition data is shown in Table 3.

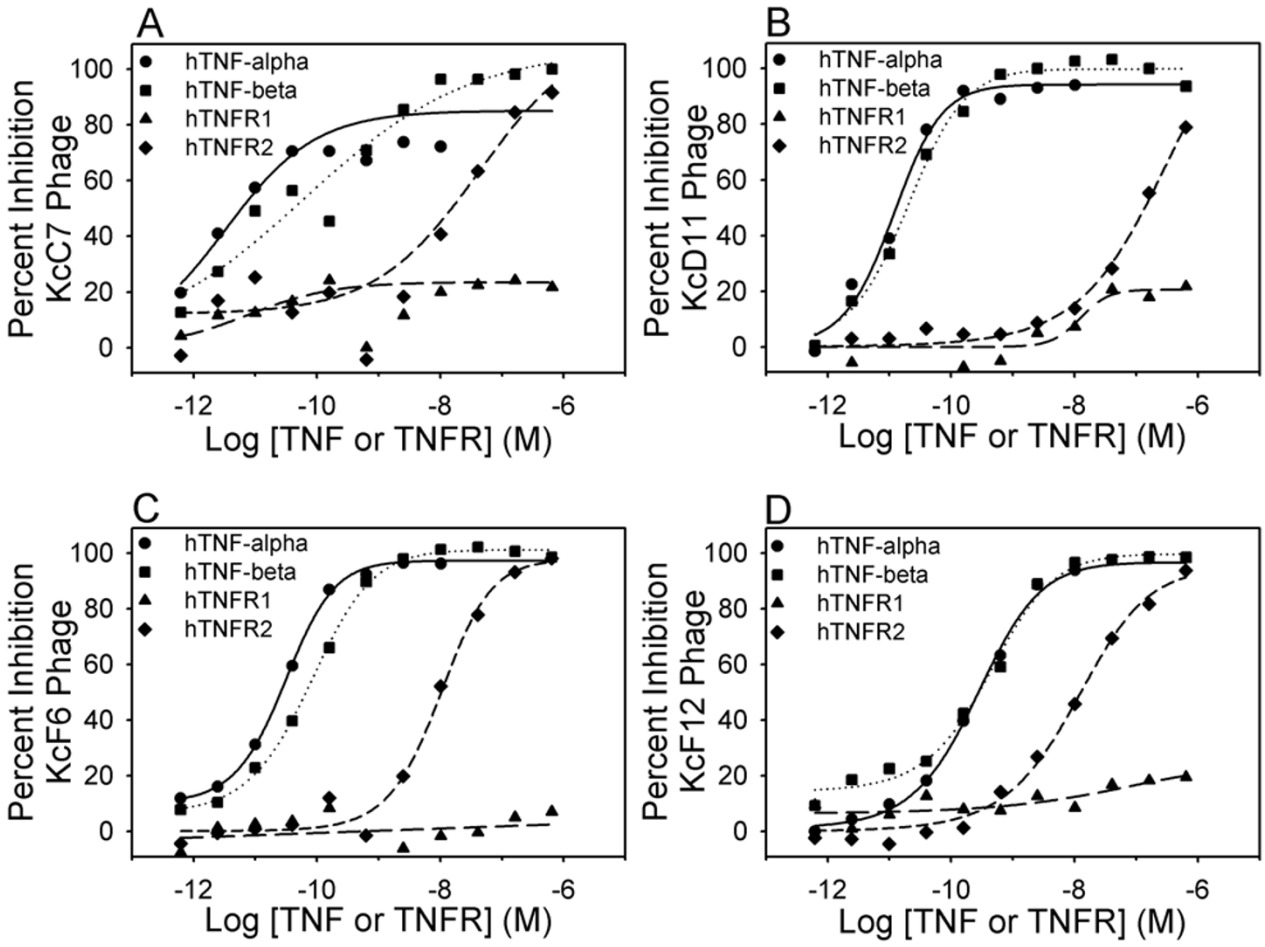

Figure 1

Competitive Displacement Of Phage Clones Expressing hTNFR2-Hotspot Peptide Ligands By Human TNF (Alpha And Beta) Or Soluble TNF Receptor (R1 Or R2) Fusions. Recombinant human TNFR2 was coated on Max-iSorp 96 well plates and blocked with PBS containing 2% non-fat milk as previously described (3). Phage clones expressing Hotspot Peptide Ligands (phage clones at their ED_{50} values) are added in the presence of varying concentrations of competitor (e.g., TNF or soluble TNF receptor). Panel A) KcC7 phage competition, Panel B) KcD11 phage, Panel C) KcF6 phage, Panel D) KcF12 phage. IC_{50} values are determined by probing for phage binding (using HRP labeled anti-M13 antibody) to TNFR2 in the presence of competitor. Data is presented as percent inhibition and to determine binding kinetic parameters data were fit to a four-parameter logistic equation using Xlfit™, an add-in for Microsoft Excel.

Binding of KcF12 to native TNFR2 was measured in Jurkat and Jiyoye cells using a radioreceptor assay. Jurkat cells have been reported to have approximately equal numbers of TNFR1 and TNFR2 per cell [27] whereas Jiyoye cells express mainly TNFR2 (unpublished data). KcF12 inhibits TNFα binding to Jiyoye cells with a Ki of about 2 uM whereas the Ki for the peptide in Jurkat cells was found to be >30 uM (Figure 3). Since TNFα binding to Jurkat probably involves both TNFR1 and TNFR2, the data offers additional confirmation of KcF12's selectivity for TNFR2.

It has been previously reported that TNFα induced a TNFR2-specific proliferation of the neuroblastoma cell line SK-N-BE [30]. KcF12, KcC7 and KcF6 inhibited the proliferative response to TNF-α in this cell line indicating that they could act as antagonists (data not shown). In addition, the same three peptides were found to be cytotoxic

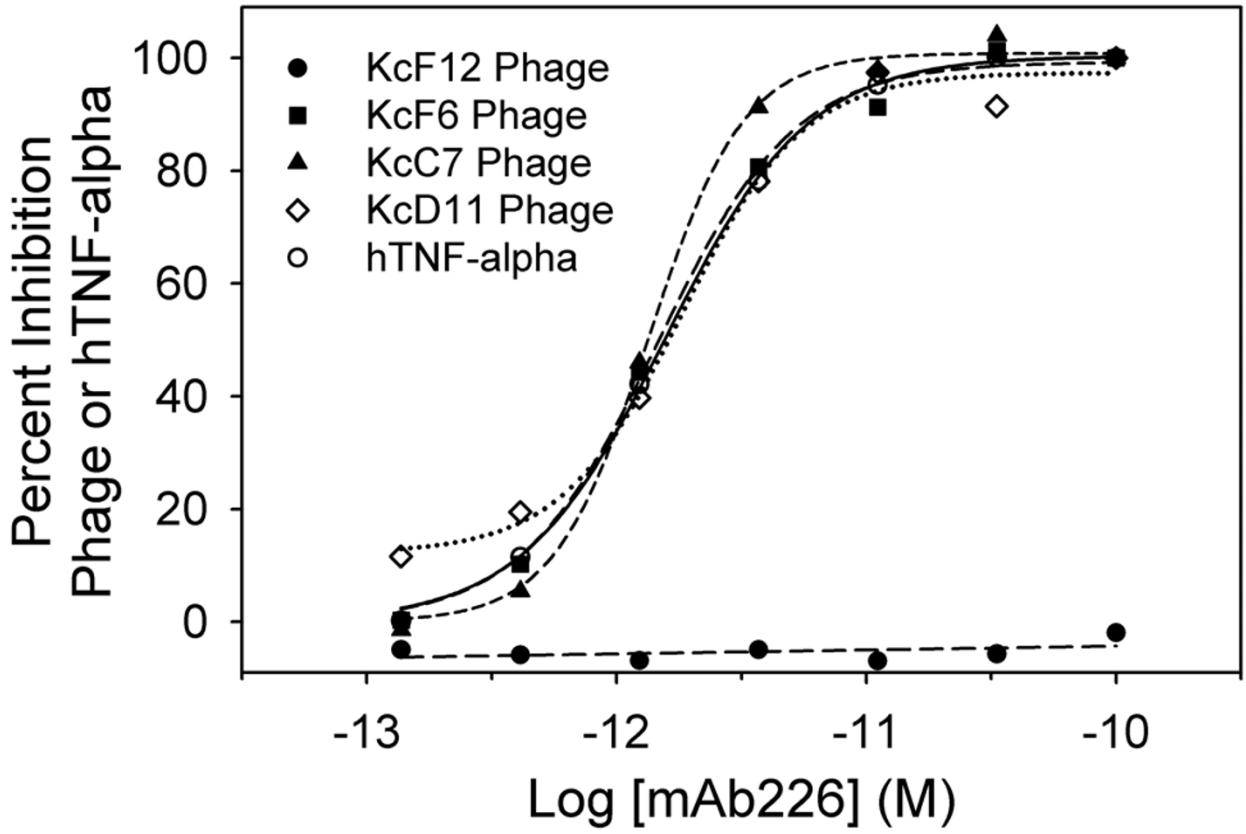

Figure 2
Inhibition Of Hotspot Peptide Ligand(s) Binding To Recombinant Human Soluble TNFR2 By Anti-TNFR2 Antibody, mAb226. HotSpot Peptide Ligand phage clones were added to 96 well plates with immobilized TNFR2 at a concentration which allowed 50% occupancy of receptor binding sites. Anti-TNFR2 antibody, mAb226, was added and serially diluted for competition. To visualize receptor bound HSPL-phage the reaction was probed with HRP labeled anti-M13 antibody. To demonstrate mAb226 interacted with the TNF binding pocket, TNF-alpha was added in a manner as described for the phage clones and TNF binding was determined using an anti-TNF antibody followed by HRP-anti-mouse IgG antibody. Data is presented as percent inhibition and were fit to a four-parameter logistic equation using Xlfit™, an add-in for Microsoft Excel.

to SK-N-BE cells in the absence of TNFα (Figure 4a). KcF12 possessed the strongest killing activity with an IC_{50} of ~700 nM whereas the IC_{50}s for KcC7 and KcF6 were 2 uM and 7 uM, respectively. None of the peptides induced a cytotoxic response in cell lines not expressing TNFR2 (data not shown). Interestingly, KcD11, which shares a motif with KcF6 (Table 1), had no effect on SK-N-BE cells. The absence of activity by KcD11 did not appear to be due to breakdown of the peptide during culturing since mass spectral analyses on aliquots retrieved from culture media showed relatively no degradation of peptides between the initial and the final time points (data not shown). Therefore, the proliferation data suggests evidence of a hotspot on TNFR2 recognized by KcF12, KcC7 and KcF6 that is linked to this unique biological effect. The inhibition of the KcC7 and KcF6 cytotoxic effect with mAb226 (Figure 4b) confirms the relationship between the peptide-induced cytotoxic event and TNFR2 because of the antibody's specificity for TNFR2. Overall, these data indicate

Table 2: Phage Competition ELISAs

	Competitor IC_{50} (M)			
Reporter Ligand	hTNF-alpha	hTNF-beta	hTNFR1	hTNFR2
KcC7 phage	3.30E-12	6.29E-11	+/-	4.25E-08
KcD11 phage	1.14E-11	1.93E-11	+/-	2.08E-07
KcF6 phage	3.01E-11	7.93E-11	0	9.87E-09
KcF12 phage	2.60E-10	4.23E-10	+/-	4.17E-08

Phage-expressed HSPLs were added in the presence of varying concentrations of competitor (e.g., TNF ligands or soluble receptors). IC_{50}s are determined by probing for phage binding to target in the presence of competitor.

Table 3: Summary Of HotSpot Ligand Interactions With TNFR2

Phage	Size (aa)	Motif	Competed by TNF-alpha & TNF-Beta	Competed by Peptides	Competed by Anti-TNFR2 (mAb226)	Antagonism	Cytotoxic Response On SK-N-E cells	Cytotoxic Response Inhibited by
KcF7	20	No	+	+	+	+	+	mAb226
KcD11	20	Yes	+	+	+	-	-	NA
KcF6	20	Yes	+	+	+	+	+	mAb226
KcF12	40	No	+	+	-	+	+	KcD11

NA: Not applicable

that the surface topology of the TNFR2 binding site is very complex and may be composed of multiple hotspots capable of different regulatory functions.

Discussion

Peptide libraries have been successfully used for identifying novel peptides mimicing natural molecules in terms of the critical protein:protein interactions involved in biological activity. Yanofsky et al. [31] described a peptide antagonist to IL-1 that was active in the nM range and Wrighton et al. and Livnah et al. [32,33] identified peptides binding to the erythropoietin (EPO) receptor with full agonist activity *in vivo*. Neither the EPO agonist nor the IL-1 antagonist peptides show any significant sequence homology to the natural ligand suggesting that they act as mimics of the natural ligand. In the case of the EPO agonists, X-ray crystallographic analysis revealed that one of these peptides spontaneously formed non-covalent homodimers that allowed the dimerization of two EPO receptors. Two families of small peptides binding to the human thrombopoietin receptor (TPOR) were isolated that competed with the natural ligand TPO [34]. The peptide with the highest affinity was subsequently dimerized and shown to function as a potent TPO agonist *in vivo*. Recently, a small, non-peptide organic molecule binding to one chain of the EPO receptor was multimerized and shown to mimic the biological activities of the EPO [35].

In this report we show that HSPLs can be isolated that bind to human TNFR2 at its ligand-binding site and subdivide it into multiple hotspots. All of the peptides were competed by both TNFα and TNFβ implying they recognized a hotspot that mapped either within or at least overlapping the ligand-binding domain of TNFR2. The HSPLs KcC7, KcD11, KcF6 but not KcF12 were also inhibited by the neutralizing antibody mAb226 suggesting the 20 mers but not KcF12 interact with a hotspot overlapping with the one recognized by antibody. The fact that recombinant KcF12 peptide competed all of the phage expressed peptides (Table 3) indicated that the four peptides also recognize a common hotspot which may be the one responsible for TNF ligand binding to the receptor. In addition, the biological data suggests that KcD11 binds to a hotspot that can be further separated from KcF12, KcF6 and KcC7. While we cannot rule out possible allosteric interactions between the peptides, the data suggests that the HSPLs were able to dissect the ligand-binding region of TNFR2 into several structural and/or functional hotspots. In addition, the HSPLs were specific for TNFR2 unlike the native ligands TNF-α and TNF-β which bind to both receptors. These results demonstrate that the approach has the ability to dissect critical protein:protein interaction domains in terms of both structural and functional elements. Using a similar approach, we recently reported on

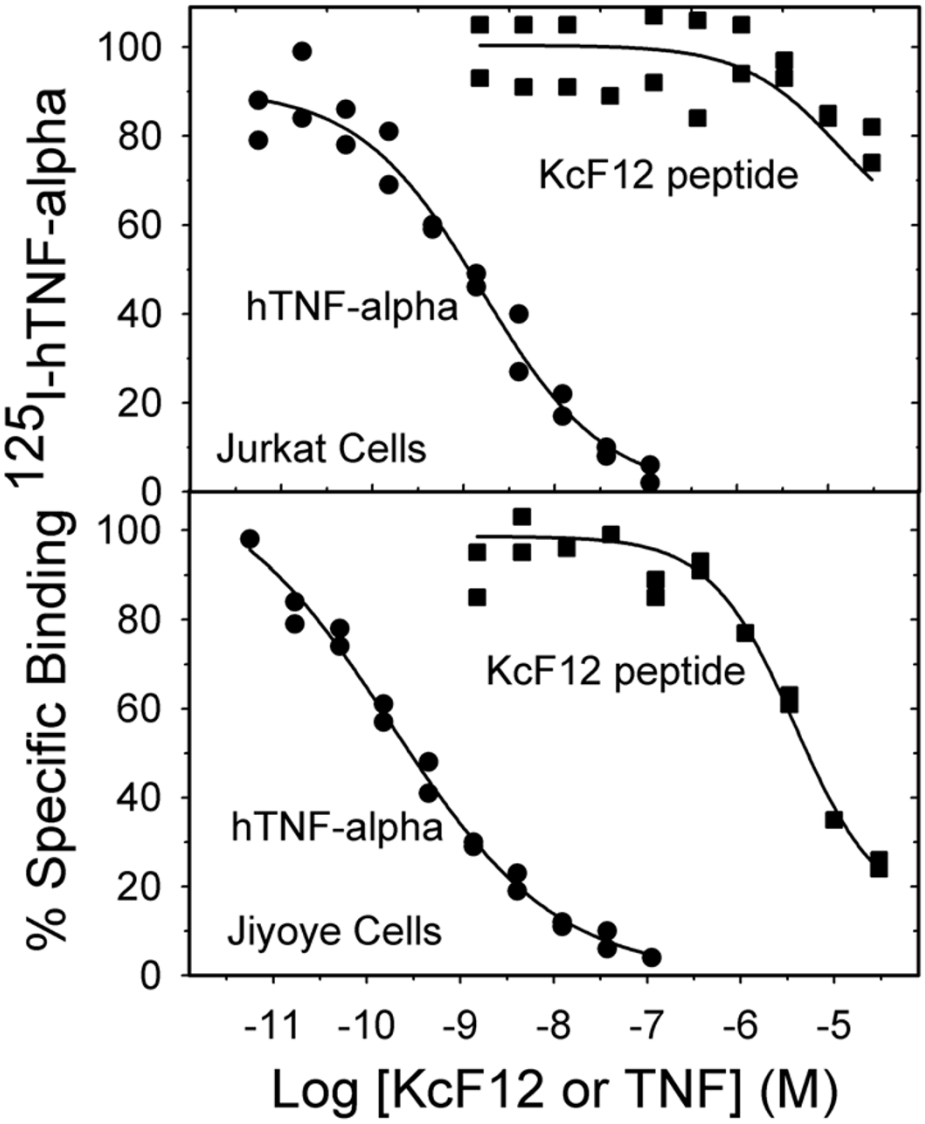

Figure 3

Hotspot Peptide Ligand Kcf12 Competes TNF Binding To Endogenous TNFR2 Receptors. The data are from representative TNFα and KcF12 competition experiments in Jurkat and Jiyoye cells. The binding of TNFα to TNFR2 on intact cells was measured in a radio-receptor assay using Jurkat and Jiyoye cells and the MultiSreen™ assay filter plate system as described [39]. Data analysis was performed with an add-in for Microsoft Excel, XIfit™ (ID Business Solutions, Guildford, UK), which allows curve fitting using nonlinear regression. To determine binding kinetic parameters, the four-parameter logistic equation y = Bottom + (Top – Bottom)/(1 + $(IC_{50}/x)^{Hill\ slope}$) was used to calculate IC_{50} values and Hill slopes. K_i values were calculated according to the equation, $K_i = IC_{50}/(1 + [ligand]/K_d)$. Data from replicate experiments in Jurkat cells (upper panel) yielded IC_{50} value of 1.64 ± 0.62 × 10^{-9} M (mean ± SD, n = 4) with a Hill slope of -0.66 ± 0.07 for TNF-alpha. Data from replicate experiments in Jiyoye cells (lower panel) yielded IC_{50} value of 2.28 ± 0.96 × 10^{-10} M (mean ± SD, n = 4) with a Hill slope of -0.52 ± 0.03 for TNF-alpha. Data from replicate experiments in Jiyoye cells (lower panel) yielded IC_{50} values of 3.56 ± 0.35 × 10^{-6} M (mean ± SD, n = 4) for HotSpot TNFR2 peptide KcF12.

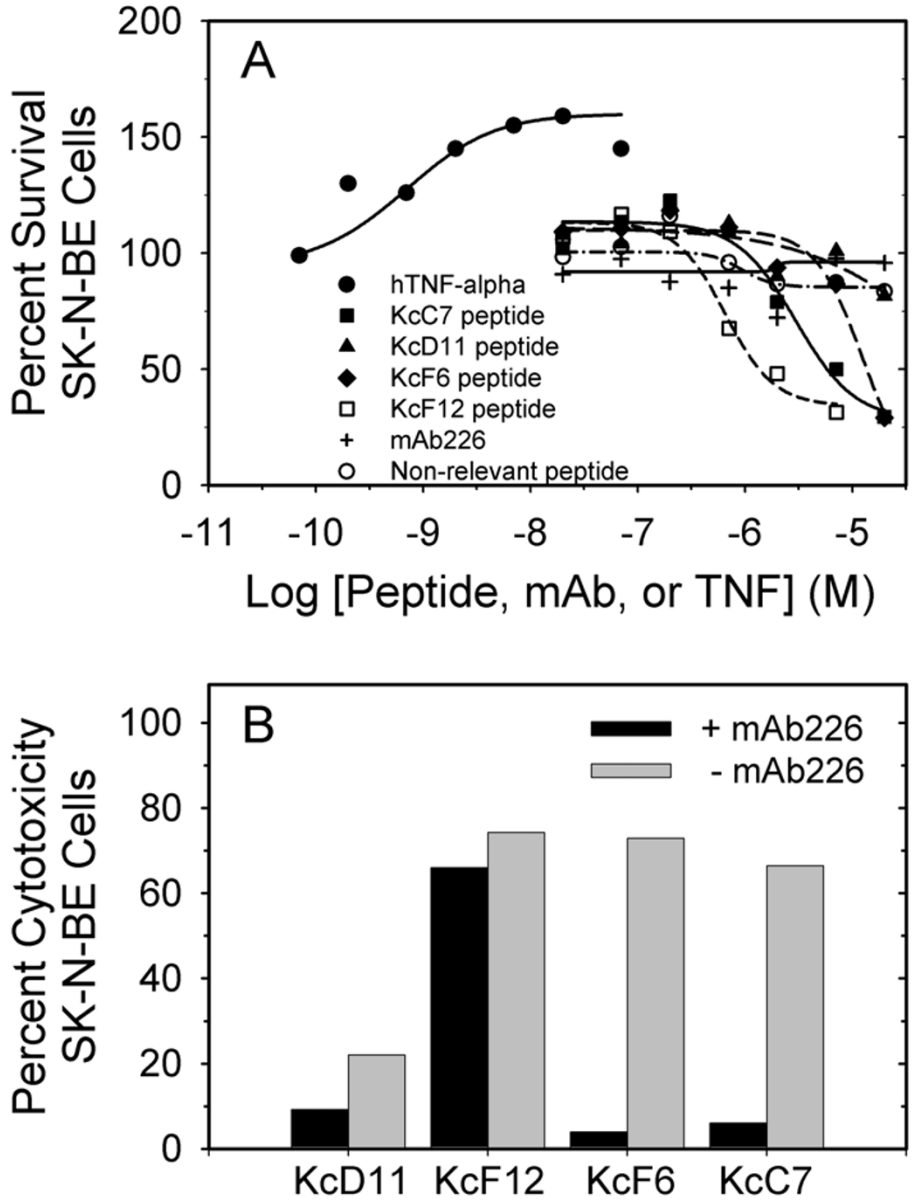

Figure 4

Cytotoxic Effect induced By The HotSpot Peptide Ligands In SK-N-BE Cells. SK-N-BE cells were seeded in 96 well plates using SK-N-BE medium and allowed to grow to 75–80% confluency. Cells were starved overnight in media containing 1% serum before each cell experiment. Briefly, cells were first treated with a TNFR1 specific receptor blocking antibody, mAb225 (10 μg/mL), for at least 30 min at 37°C prior to the addition of test agents. All agents used in the cell-based experiments were serially diluted and mixed in a separate 96 well plate before transferring into the 96 well plate containing SK-N-BE cells. Cells were then returned into the incubator for 40–45 hrs before the addition of 0.1 vol. of WST-1 (Roche Diagnostics Corporation, Indianapolis, IN) to quantitate cell number. The plates were further incubated and developed according to the manufacturer's instruction. Panel A) Synthetic HotSpot peptide ligands, KcF12, KcC7, and KcF6 act as inverse agonists by inhibiting TNFR2-linked cellular survival in SK-N-BE cells. TNF-alpha causes cellular proliferation via a TNFR2-linked mechanism in SK-N-BE cells. Addition of a control peptide with a non-relevant sequence does not alter cell survival. Panel B) Blockade of TNFR2 binding pocket by Anti-TNFR2 antibody, mAb226, inhibits the cytotoxic action of KcC7 and KcF6, but not KcF12. Peptides were added at their ED_{80} values while mAb226 was at 10 μg/mL. IC_{80} (10 μM) concentrations, while KcD11 was added at either 10 μM or 100 μM.

the subdivision of the ligand binding domain on the insulin receptor into two separate and non-overlapping hotspots with different biological activities [3].

It has been shown that TNFα induced proliferation of the undifferentiated neuroblastoma cell line, SK-N-BE, resulted from activation of TNFR2 [30]. Proliferation of SK-N-BE cells could be suppressed by the pre-incubation of cells with anti-TNFR2 blocking antibodies without concomitant cytotoxicity (data not shown). While all of the peptides blocked the agonist effects of TNFα indicating that they can act as antagonists, KcC7, KcF6 and KcF12 by themselves induced a cytotoxic response by killing SK-N-BE cells in the absence of TNFα. This is surprising because these peptides share a hotspot with the natural ligand TNFα (which is proliferative rather than-cytotoxic) and KcC7 and KcF6 bind to a hotspot common to the neutralizing but non-cytotoxic, antibody mAb226. Overall, these data suggest that activation (agonism or cytotoxicity) of TNFR2 is mediated through unique hotspots on the receptor that can be defined by the peptides. Under the present experimental conditions, it is unclear whether the natural ligand, TNFα, touches the hotspot responsible for cytotoxicity although one can speculate that, if it does, the cytotoxic effect might be nullified by the sum of the natural ligand:receptor interactions. Competition experiments showing that mAb 226 reversed the effects of KcC7 and KcF6 confirm that these two peptides induce their effects though their interaction with TNFR2. These findings suggest that TNFR2 has multiple biological functions. In addition to transferring TNFα to TNFR1 as proposed by the ligand passing model [36], TNFR2 may have a role in modulating proliferative responses in both positive and negative manner. Ongoing studies will attempt to elucidate the mechanism of action behind the phenomenon such as the effects on downstream signaling pathways including NFkB, and apoptosis. However, it is possible that peptide-induced cytotoxicity works through a mechanism similar to TNF mediated apoptosis in T-cells [37].

Conclusions

In summary, HSPLs were isolated with which we were able to dissect the ligand-binding domain of a natural receptor and generate information about novel and unexpected bioactivities. This technology has also been successfully used for known protein targets, such as the insulin receptor [3] and TNF-β [38] as well as non-protein targets such as disease-specific RNAs [38]. We have also shown that the HSPL libraries identify hotspots on novel genes of unknown function and identify functional interaction pathways (manuscript in preparation). Overall, the use of HSPLs have the potential to become a general approach for scanning and understanding complex protein:protein interactions.

Methods

Materials

E. coli, strain TG1 (genotype = K12Δ*(lac-pro)*, *supE*, *thi*, *hsdΔ5/F' [traD36*, *proAB*, *lacI*^{q}, *lacZΔM15*]) was obtained from Pharmacia (Piscataway, NJ). Recombinant human Tumor Necrosis Factor-α (rhTNFα) was purchased from R&D Systems, Inc. (Minneapolis, MN) or PeproTech Inc. (Rocky Hill, NJ). Monoclonal antibodies against recombinant human soluble TNF receptor RI (mAb225) and RII (mAb226) were purchased from R&D Systems, Inc. (Minneapolis, MN). WST-1 was purchased from Roche (Nutley, NJ). ^{125}I-TNFα (NEX 257) with a specific activity of 97 μCi/μg (1698 Ci/mmol) was purchased from NEN Life Science Products, Inc (Boston, MA). Beckman Ready Safe™ scintillation fluid was purchased from Beckman Coulter, Inc. (Fullerton, CA). MultiSreen™ assay filter plate system and 96-well MultiScreen® MAFC filter plates were from Millipore (Bedford, MA). RPMI 1640 media, fetal bovine serum, and GlutaMAX™-I were obtained from Life Technologies (Rockville, MD). Polyvinylpyrrolidone (PVP), Tween-20R™, phosphate-buffered saline (PBS), and Dulbecco's phosphate-buffered saline (containing no calcium or magnesium) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents used were analytical grade.

Cell Culture

SK-N-BE neuroblastoma cells, Jurkat (TIB-152) and Jiyoye (CCL-87) cells were obtained from the ATCC (Manassas, VA). SK-N-BE and Jurkat cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and 2 mM GlutaMAX™-I, while Jiyoye cells were maintained in RPMI 1640 media supplemented with 15% FBS and 2 mM GlutaMAX™.

Design of the Primary Peptide Libraries

A description of library design and production has been previously published [3,28].

Subtractive Panning and ELISA

Panning was done as described [3,28], except that only 6 wells of a 96-well microtiter plate were coated with target at concentrations ranging from 10–100 ng/well. The cross reactive phages from the eluted phage were then subtracted by panning sequentially against human TNFR1, TNFα and TNFβ as a way to obtain peptides specific to the original target. ELISA was done as previous described [3,28].

Phage Competition ELISA Studies

Target was coated on MaxiSorp plates and blocked with NFM-PBS as described [3,28]. HSPLs (phage clones or synthetic peptides) are added in the presence of varying concentrations of competitor (e.g., TNF ligand or soluble receptor). IC_{50}s are determined by probing for phage or peptide binding to target in the presence of competitor.

Receptor Binding and Competition Assay

The binding of TNFα to TNFR2 on intact cells was measured in a radioareceptor assay using Jurkat and Jiyoye cells and the MultiSreen™ assay filter plate system as described [39]. Data analysis was performed with an add-in for Microsoft Excel, Xlfit™ (version 2.0) from ID Business Solutions (Guildford, UK), which allows curve fitting using nonlinear regression. To determine binding kinetic parameters, the Four-Parameter logistic equation y = Bottom + (Top – Bottom)/(1 + $(IC_{50}/x)^{Hill\ slope}$) was used to calculate IC_{50} values and Hill slopes. K_i values were calculated according to the equation, $K_i = IC_{50}/(1 + [ligand]/K_d)$, as described by Cheng and Prusoff [40].

Proliferation cell assay

SK-N-BE cells were seeded loosely in 96 well plates in SK-N-BE medium and allowed to grow to 75–80% confluency. Cells were starved overnight in media containing 1% serum before each cell experiment. Briefly, cells were first treated with a human TNFR1 blocking mAb, mAb225 (10 ug/ml), for at least 30 min at 37 C prior to the addition of agents. All agents used in the cell experiment were serial diluted at 1:2 and mixed in a separate plate before transferring into the actual cell plate. The starting highest final concentration of each peptide was at 30 uM or at its highest possible concentration. Cells were then returned into the incubator for 40–45 hrs before the addition of 1/10 vol of WST-1. The plates were further incubated and developed according to the manufacturer's instruction.

Authors' Contributions

KcH isolated and characterized the HSPLs binding to TNFR2. RB prepared the primary and secondary HSPL libraries. PW and ML produced and purified the recombinant peptides and performed the stability studies with the peptides. PF and VR did the binding assays. AB contributed helpful suggestions about the design of the studies. NG conceived the study and participated in the design and coordination of the experiments. All authors have read and approved the final manuscript.

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