Chemistry & Biology
Article

Decoding a PNA Encoded Peptide Library by PCR: The Discovery of New Cell Surface Receptor Ligands

Nina Svensen,[1] Juan José Díaz-Mochón,[1] and Mark Bradley[1,*]
[1]School of Chemistry, University of Edinburgh, Joseph Black Building, Edinburgh EH9 3JJ, UK
*Correspondence: mark.Bradley@ed.ac.uk
DOI 10.1016/j.chembiol.2011.07.017

SUMMARY

The ability to screen and identify new ligands for cell surface receptors has been a long-standing goal as it might allow targeting of pharmaceutically relevant receptors, such as integrins or G protein coupled receptors. Here, we present a method to amplify hits from a library of PNA-tagged peptides. To this end, human cells, overexpressing either integrins or the CCR6 receptor, were treated with a 10,000 member PNA-encoded peptide library. Extraction of the PNA tags from the surface of the cells was followed by a PNA-tag to DNA translation and amplification enabling decoding of the tags via microarray hybridization. This approach to ligand discovery facilitates screening for differences in surface-receptor ligands and/or receptor expression between different cell types, and opens up a practical approach to PNA-tag amplification.

INTRODUCTION

Numerous tagging strategies for combinatorial libraries have been developed with perhaps the most commonly applied approach at this time being DNA-encoding (Franch et al., 2007; Wrenn et al., 2007; Clark et al., 2009; Buller et al., 2009). The advantages of nucleic acid encoding are manifold, including the opportunities for massively parallel and quantitative decoding, either by the application of next generation sequencing or microarray approaches, which allow not only the identification and relative quantification of ''hits'' but also the generation of structure-activity relationships (Brenner and Lerner, 1992; Needels et al., 1993; Nielsen et al., 1993; Ben-Dor et al., 2000; Melkko et al., 2004; Gartner et al., 2004; Morgan et al., 2005; Scheuermann et al., 2006).

PNA is a DNA mimic, which offers great resistance to biological degradation while having a high binding affinity for DNA, factors that render PNA a valuable encoding alternative to DNA (Winssinger et al., 2001, 2002; Díaz-Mochón et al., 2005; Harris and Winssinger, 2005; Urbina et al., 2006; Debaene et al., 2007; Pianowski and Winssinger, 2008) especially for in vivo applications (Demidov et al., 1994; Nielsen, 2004). As such, PNA-encoding of small molecules has allowed investigation of complex biological targets for the identification of specific substrates for proteases (Winssinger et al., 2001, 2002, 2004; Urbina et al., 2006; Díaz-Mochón et al., 2006) and tyrosine kinases (Díaz-Mochón et al., 2005) as well as the identification of cell penetrating homing peptides (Svensen et al., 2011a). However, a classic downside to PNA-encoding is that PNA cannot be amplified by standard nucleic acid amplification techniques (Nielsen, 2004). Thus, if a screen yields a low concentration of PNA-tags, which are below those needed for microarray analysis, then the screen would fail unless a method could be developed for amplification of the PNA-tags.

Herein we report the screening of a 10,000 member PNA-encoded peptide library (Library-1; Figure 1) (Pouchain et al., 2007; Svensen et al., 2011b) with live cells and the decoding of the attached ligands via indirect DNA amplification of the PNA-tags with subsequent microarray analysis. Decoding was achieved via hybridization of the isolated PNA with a 10,000 member single stranded (ss) DNA library complementary to the 10,000 members of the PNA library followed by degradation of non-hybridized ssDNA. Asymmetric PCR amplification of the DNA (protected by the PNA) with a fluorescent primer generated ssDNA, which could be analyzed using DNA microarrays (Figure 1). This allowed information on the interaction between the peptide ligands and the cell surface receptors to be extracted on a spot-by-spot (compound-by-compound) basis.

RESULTS AND DISCUSSION

Amplification of PNA Signal

Library-1 (Svensen et al., 2011b) designed to bind integrins was based on the peptide sequence reported by Kuratomi et al. (1999) and consisted of 10,000 nonapeptide-PNA conjugates with four variable positions each containing 10 different amino acids: Ac-Phe-Gln-AA_4-AA_3-Tyr-AA_2-AA_1-Ile-Lys-PNA_{17}-fluorescein with each variable amino acid encoded by a PNA-triplet (Figure 1) (Pouchain et al., 2007). This library (10 μM, corresponding to 1 nM of each library member) was incubated with the cell lines D54 (overexpressing $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins) (Gomez-Manzano et al., 1996; Fueyo et al., 1998) and HEK293T-CCR6 (overexpressing CCR6) (Zaballos et al., 1996). Following incubation and washing, pronase degradation of extracellular receptor moieties as well as any bound peptide ligands allowed release of the PNA tags (corresponding to peptides attached to the cell surface). Due to the limited number of cells and receptors expressed on the cell surface the amount of purified PNA was

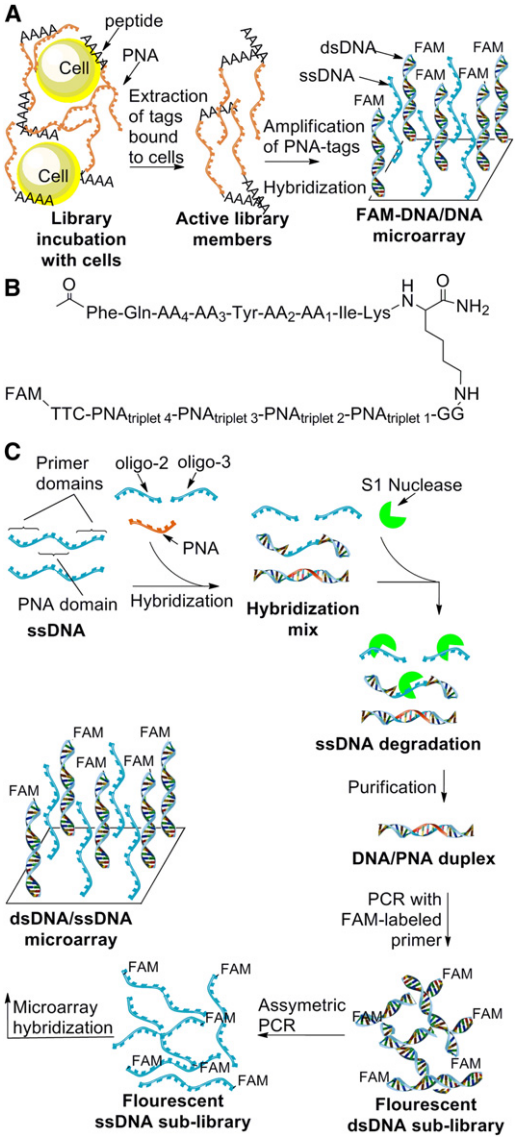

Figure 1. Screening 10,000 PNA-Encoded Peptide Ligands and PNA-Tag Amplification

(A) The general strategy for the identification of ligands for cell surface receptors by microarray analysis of a library of PNA-encoded peptide ligands.

(B) Generic structure of Library-1. AA_1, AA_2, and AA_4 were Ile, Val, Phe, Pro, Arg, Glu, Lys, *d*-Pro, Ser or *d*-Val and AA_3 Ile, Val, Phe, Ala, Pro, Arg, Glu, Lys, *d*-ALa, Ser, or Pro. FAM is 5(6)-carboxyfluorescein amide.

(C) The strategy of indirect amplification of the PNA-tags. The PNA extracted from cells was hybridized with a complementary ssDNA library (ssDNA-Library-1) and unhybridized ssDNA was degraded with a single-strand specific nuclease. The PNA/DNA duplexes were amplified by PCR to produce fluorescently labeled ssDNA analogous to the PNAs extracted from cells. The fluorescently labeled ssDNA was hybridized to a complementary microarray to allow decoding of the amplified ssDNA (and hence the PNA-tags and their encoded peptides).

See also Table S1.

less than required for microarray analysis (see Supplemental Information available online), and an approach was therefore developed, which involved indirect amplification of the PNA-tags (Figure 1).

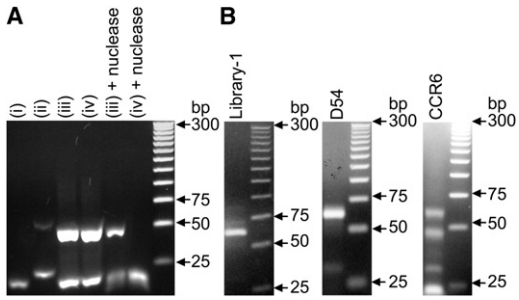

Figure 2. DNA Gel Electrophoresis of the Amplified PNA

(A) Proof of concept of PNA amplification. (i) oligonucleotides-2 and -3; (ii) oligonucleotide-1; (iii) dsDNA/PNA complex; (iv) oligonucleotides-1, -2 and -3 (ssDNA/dsDNA hybrid complex).

(B) Library-1: Control showing the PCR amplified 10,000 member PNA-Library-1 (ssDNA-Library-1-FAM); D54: FAM-labeled ssDNA decoded library (ssDNA-D54-FAM); CCR6: FAM-labeled ssDNA decoded library (ssDNA-HEK293T-CCR6-FAM).

See also Figure S1.

Ten thousand complementary ssDNA oligonucleotides (ssDNA-Library-1) (Supplemental Information) were generated by direct amplification "off" a DNA microarray and this was hybridized to the recovered PNA and to oligonucleotide-2 and -3 (Svensen et al., 2011c) (Figure 1; Table S1). This was followed by nuclease degradation of the ssDNA (S1 nuclease) and purification of the dsDNA/PNA complexes by gel column filtration (Figure 1). Because the isolated PNA-tags protected their complementary DNAs from S1 nuclease degradation, only DNA complementary to the PNA tags was available for PCR amplification and subsequent microarray analysis. Oligonucleotide-2 and -3 served to protect the primer binding domains of the DNA from nuclease degradation (Figure 1). The purified dsDNA/PNA complexes were then amplified by asymmetric PCR using a FAM-labeled primer (Table S1) producing FAM-labeled ssDNA decoded libraries (ssDNA-D54-FAM, and ssDNA-HEK293T-CCR6-FAM) (Figure 2). As a positive control the ssDNA-Library-1 was hybridized with the 10,000 member PNA-Library-1 as well as oligonucleotides-2 and -3, which was followed by nuclease degradation and asymmetric PCR amplification as described above, generating a 10,000 member FAM-labeled ssDNA decoded library (ssDNA-Library-1-FAM) (Figure 2).

This approach was initially validated by hybridization of a single DNA oligonucleotide (oligonucleotide-1, [ii]; [Table S1] with a strand of PNA [TGTTTGTTTGTT] [Svensen et al., 2008]) as well as oligonucleotides-2 and -3 ([i]; Table S1) to give a double stranded DNA/PNA duplex (Figures 1 and 2). This hybridization reaction was treated with a single-strand specific DNase (Figure 2, [iii]). A negative control hybridization (of oligonucleotides-1, -2, and -3) and nuclease digestion were carried out in the absence of protective PNA resulting in complete DNA degradation illustrating that the DNA protected PNA can be selectively amplified (Figures 1 and 2, [iv]).

Microarray Hybridization and Deconvolution of the Data

The DNA microarrays used contained 4 × 44,000 features with four copies of each library member and 4000 controls on each 44 K subarray. The FAM-labeled ssDNA decoded libraries

Table 1. Identified "Hit" Peptides

Peptides
FAM-Ahx-FQSIYPpIK-NH_2 = (v)
FAM-Ahx-FQIPYIIIK-NH_2 = (vi)
FAM-Ahx-RGD-NH_2 = (vii)
Rho-Ahx-RGD-NH_2 = (viii)

Ahx, aminohexanoic acid; p, d-Pro.

were hybridized to a DNA-microarray complementary to the 17 bp PNA encoding domains. Microarray imaging and analysis was used to extract the intensity of the FAM-label, thereby allowing the relative amount of PNA extracted from the cells and hence the peptides binding to the cells to be determined. Data analysis allowed identification of the top 100 binding peptides from the 10,000 member library (Supplemental Information).

Scatter plots of the relative cellular-peptide binding affinity for the top 100 peptides versus the variable amino acids for positions AA_4 to AA_1 were constructed (Figure S1). This allowed identification of the most preferred amino acids for each of the variable positions (i.e., the amino acids with the highest number of high-intensity peptides): FQSIYPpIK and FQIPYIIIK for D54, and HEK293T-CCR6 cells, respectively.

Synthesis of "Hit" Peptides

To verify and quantitatively compare cellular affinities of the "hit"-peptides (Figure S1) the fluorescein-labeled peptides, FAM-Ahx-FQSIYPpIK-NH_2 [Table 1, (v); p = *d*-Pro and Ahx = aminohexanoic acid] and FAM-Ahx-FQIPYIIIK-NH_2 [Table 1, (vi)] were synthesized (Díaz-Mochón et al., 2005; Svensen et al., 2008). As a positive control for integrin binding the fluorescein and rhodamine-labeled peptides, FAM-Ahx-RGD-NH_2 [Table 1, (vii)] and Rho-Ahx-RGD-NH_2 [Table 1, (viii)] were also synthesized.

Peptide-Cell Binding Is Peptide-Specific

D54, HEK293T, and HEK293T-CCR6 cells were analyzed by flow cytometry following incubation with the FAM-labeled peptides or with a CCR6 antibody (anti-human CCR6). D54 cells bound the identified integrin peptide ligand (v) as evidenced by induction of a significant shift in the level of fluorescence compared to that of the untreated cells (Figure 3; Figure S2). Furthermore, this shift was similar in magnitude to that induced by the positive control (vii; at the same concentration) showing that these peptides have a similar affinity. Treatment with pronase/EDTA totally removed the fluorescence signal of peptide (vii) demonstrating that the peptide is associated with the cell surface (Figure 3; Figure S2). Incubation with (viii) and (v) at equimolar concentrations reduced (v) binding by 55% and (viii) binding by 57%, whereas (viii) binding was reduced by 80% following the addition of 10 equivalents of (v), illustrating that binding is integrin mediated.

In HEK293T-CCR6 cells, the identified peptide (vi) induced a significant shift in the level of fluorescence compared to that of the untreated cells and this was slightly smaller than that induced by the FAM-labeled CCR6 antibody. Again treatment with pronase/EDTA reduced the fluorescence signal of the peptides

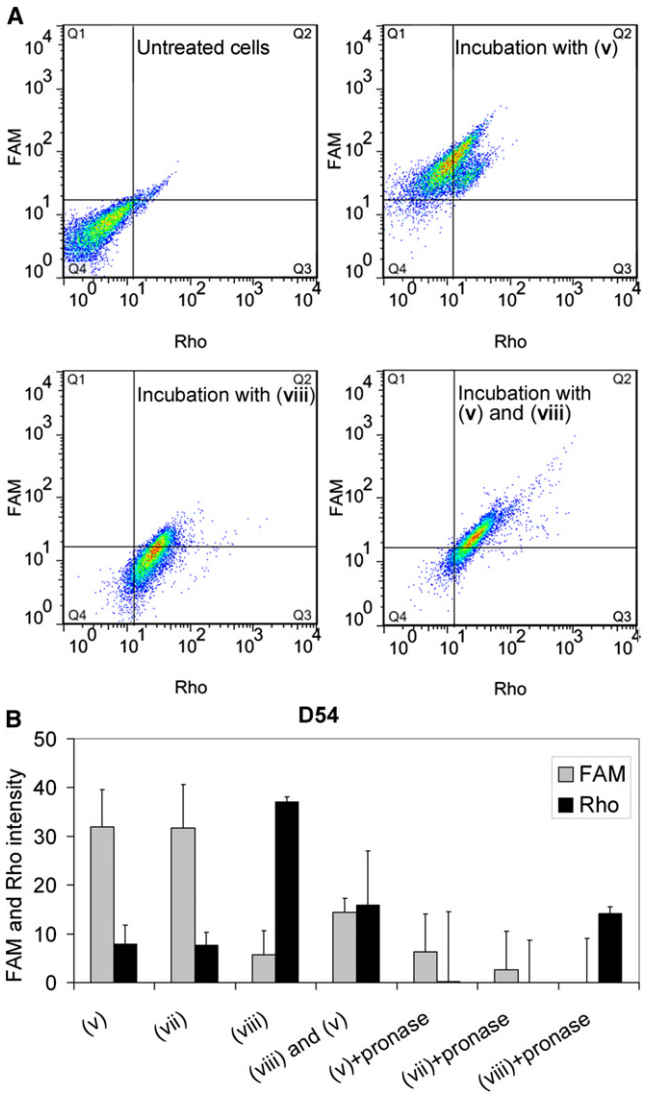

Figure 3. Flow Cytometry Analysis of Peptides Binding to D54 Cells

(A) FAM versus Rho-filtered scatter plots gated for live, single cells following incubation with (v), (vii), and (viii) using a FACS Aria cytometer with FITC and PE-Texas-Red filters (10,000 populations, n = 3). D54 cells were detached by scraping for minimal destruction of the extracellular receptor moieties or by trypsin/EDTA as a control for extracellular binding of peptides.

(B) Mean FAM and Rho-filtered fluorescence of scatter plots versus incubation with the labeled peptides. Error bars indicate ± standard deviation (SD) (n = 3). See also Figures S2 and S3.

demonstrating that the peptides were associated with the cell surface (Figure 4; Figure S2).

Peptide-Cell Binding Is Receptor-Mediated

To verify that binding of the identified ligands was mediated by the receptor in question, a soluble inhibitor for the respective receptor, labeled with a different fluorophore than the peptides, was coincubated with the peptides. The D54 cells overexpress $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins (Gomez-Manzano et al., 1996; Fueyo et al., 1998; Coughlan et al., 2009), which can be blocked by

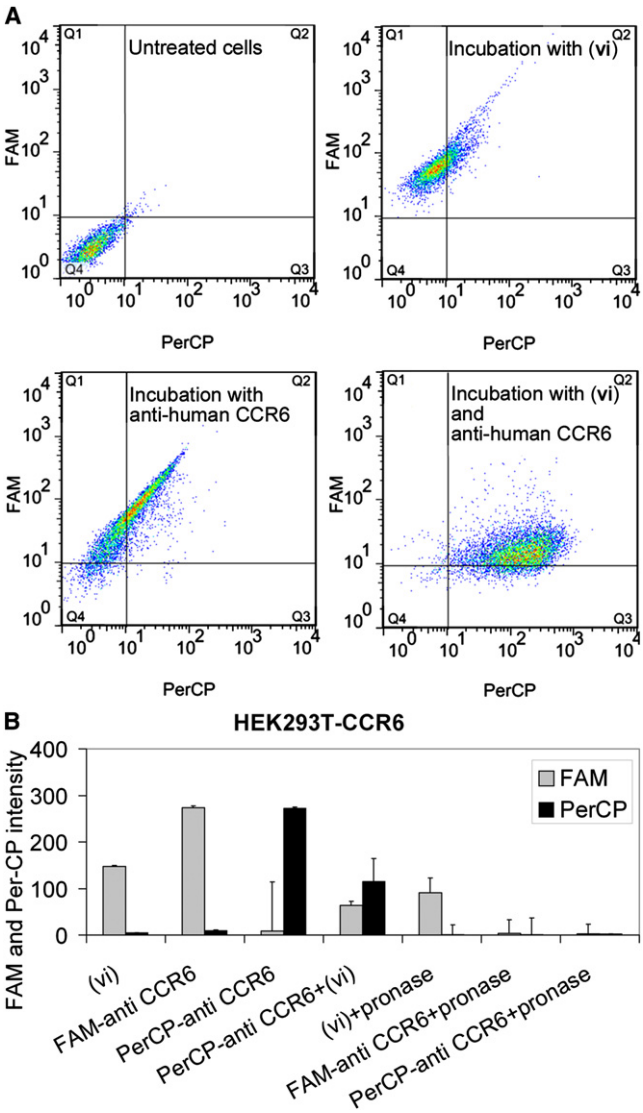

Figure 4. Flow Cytometry Analysis of Peptides Binding to HEK293T-CCR6 Cells

(A) FAM versus peridinin chlorophyll protein complex (PerCP)-filtered scatter plots gated for live, single cells following incubation with (vi) or anti-human CCR6 using FITC and PE-Texas-Red filters. Conditions as given in Figure 3.

(B) Mean FAM and PerCP-filtered fluorescence of scatter plots versus incubation with (vi) and the CCR6 antibodies. Error bars indicate ± SD (n = 3).

See also Figures S2 and S3.

the natural ligand, RGD; whereas, the CCR6 receptor can be blocked by antihuman CCR6.

Incubation with PerCP-labeled CCR6 antibody and (vi [10 μM]) reduced binding of the peptide by 56% and the antibody by 58% (Figure 4; Figure S2). Increase in peptide concentration by a factor of 10 completely blocked antihuman CCR6 binding demonstrating that (vi) binding is CCR6 mediated. As a negative control for CCR6 binding unmodified HEK293T cells were incubated with (vi) and CCR6 antibody. As expected, negligible shifts in the levels of fluorescence were induced in HEK293T cells compared to HEK293T-CCR6 cells and no competition between (vi) the CCR6 antibody was observed, which further illustrates that (vi) binding is CCR6 mediated (Figure S2).

In order for the peptides to have biological applications it is vital that they do not exhibit cytotoxicity. Cell viability upon treatment with the "hit" peptides was assessed with standard MTT (Mosmann, 1983) assays and demonstrated no cytotoxicity by the two peptides (Figure S3).

This technique for amplification of the PNA-tags allows screening of live cells (both adherent and suspension) and screening of targets in scarce amounts. As screening is carried out prior to decoding and PNA is not biologically degraded (Demidov et al., 1994; Nielsen, 2004), the technology is applicable for screening in vivo as well as in vitro.

This approach establishes a strategy for high-throughput screening for the identification of ligands for integrins and GPCRs offering an approach to de-orphanize GPCRs, for which there are no known ligands. As integrins and GPCRs are among the most heavily investigated drug targets in the pharmaceutical industry today (Miller et al., 2000; Filmore, 2004; Insel et al., 2007) identification of new agonist or antagonists of these receptors by this method could help identify new drug-targets as well as leads. The technology would also allow screening of a wide variety of other cell surface receptors and offers a tool for investigating differences in membrane receptor expression between different cell types including diseased cells.

This decoding strategy is amenable to the screening of larger libraries (e.g., containing millions of compounds), either through microarray hybridization strategies using high density arrays (such as those from Nimblegen) or via next-generation sequencing. Although it has not yet been of validated with nonpeptidic compounds, the PNA encoding strategy is sufficiently robust to enable tagging of either small molecules or peptides.

SIGNIFICANCE

Cellular incubation with a 10,000 member PNA encoded peptide library followed by PNA to DNA amplification as well as microarray and scatter plot analysis allowed consensus sequences for receptor binding (v and vi) to be extracted for $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins and CCR6 respectively. Synthesis of these "hit"-peptides followed by flow cytometry analysis verified receptor binding and demonstrated no cytotoxicity. Competition studies with the natural ligand for integrins, (vii) and an antibody for CCR6 demonstrated that binding of (v) and (vi) was $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrin and CCR6-mediated respectively.

EXPERIMENTAL PROCEDURES

Cell Culture

HEK293T, HEK293T-CCR6, and D54 cells were maintained in DMEM (Supplemental Experimental Procedures). All cells were grown in T-75 or T-25 flasks (Nunc) in high humidity and 5% CO_2 at 37°C. Cell lines were passaged by standard trypsinization (Supplemental Experimental Procedures).

Library Extraction from Cells

D54 and HEK293T-CCR6 cells were incubated with Library-1 (4 ml, 10 μM) in DMEM enriched with $CaCl_2$ (50 μM) and $MgCl_2$ (50 μM) for 30 min at 37°C. The media was removed and the cells were washed and treated with 1× pronase/EDTA in PBS for 20 min at 37°C (Supplemental Experimental

Procedures). The cells were pelleted by centrifugation, the supernatant was recovered, and the PNA-peptide conjugates were purified by filter-centrifugation affording surface bound PNA encoded peptides extracted from D54 (4.4 pmol), and HEK293T-CCR6 (2.4 pmol).

Decoding of the PNA-Tags

The PNA extracted from cells was hybridized to the complementary 10,000 member ssDNA-Library-1 as well as oligonucleotide-2 and -3 (Table S1 and Supplemental Experimental Procedures). Hereby, oligonucleotide-2 and -3 bound to the primer domains of all of the 10,000 ssDNA-Library-1 members and the PNA bound to the complementary sequences of the ssDNA-Library-1. This produced a mixture of DNA/PNA complexes: PNA/ssDNA/oligonucleotide-2 and -3 (fully double stranded) and ssDNA/oligonucleotide-2 and -3 (partly double stranded) as well as excess oligonucleotide-2 and -3 (Figures 1 and 2). The single stranded domains of the ssDNA/oligonucleotide-2 and -3 complexes and the excess oligonucleotide-2 and -3 could then be degraded by a single strand specific DNase, whereas the double stranded PNA/ssDNA/oligonucleotide-2 and -3 complexes were inert to nuclease degradation (Supplemental Experimental Procedures). Thus, the PNA/ssDNA/oligonucleotide-2 and -3 complexes comprised only the DNA strands, which were complementary to the PNA extracted from cells, whereas all other noncomplementary DNA strands had been degraded (Supplemental Experimental Procedures).

Hereafter, the ssDNA oligonucleotides in the PNA/ssDNA/oligonucleotide-2 and -3 complexes were selectively amplified by asymmetric PCR using a FAM-labeled primer (Table S1 and Supplemental Experimental Procedures), which produced FAM-labeled ssDNA libraries that were complementary to the PNA extracted from cells (ssDNA-D54-FAM and ssDNA-HEK293T-CCR6-FAM; Figure 2). As a negative control the 10,000 PNA-tags in the untreated PNA-Library-1 were also amplified in this manner, which produced a FAM-labeled ssDNA library complementary to the 10,000 member PNA-Library-1 (ssDNA-Library-1-FAM) (Figure 2; Supplemental Experimental Procedures). FAM-labeling during PCR amplification allowed subsequent microarray interrogation of the DNA libraries.

Microarray Hybridization

Library-1 complementary DNA microarrays (Oxford Gene Technology) were hybridized with the purified ssDNA-FAM (Supplemental Experimental Procedures) in 0.1% SDS in 4 × SSPE buffer (110 μl) overnight from 65°C to 27°C and the arrays were washed with 0.2% SDS in 2 × SSC for 5 min, 0.2 × SSC for 5 min, 0.1 × SSC for 5 min, and briefly rinsed in DNA grade H_2O and 10 mM Tris buffer at pH 8.0 and dried under N_2 flow. The microarrays were imaged and the images were analyzed using Bluefuse software (ArrayExpress database, accession number E-MEXP-3104; Supplemental Experimental Procedures).

Deconvolution of Microarray Data

Raw microarray data was obtained from Bluefuse (Supplemental Experimental Procedures). The average fluorescence intensity was calculated over each of the four replicates and over the noncomplementary negative control features. The average intensities were corrected for the background and normalized for the Library-1 hybridization (Supplemental Experimental Procedures).

Peptide Synthesis

Peptides were synthesized by solid-phase synthesis according to previously reported procedures (Díaz-Mochón et al., 2005; Svensen et al., 2008; Supplementary information). The peptide-PNA conjugates were cleaved from the solid support by treatment with 5% TIS in TFA (500 μl) for 2 × 2 hr and the products were precipitated with cold diethyl ether (×2) and collected by centrifugation. The peptides were purified by HPLC (Supplemental Experimental Procedures).

Flow Cytometry

HEK293T and HEK293T-CCR6 cells (Supplemental Experimental Procedures) were incubated with PerCP-anti human CCR6 (10 μg/ml), FAM-anti human CCR6 (10 μg/ml), FAM-Ahx-FQIPYIIIK-NH_2 (10 μM), as well as PerCP-anti human CCR6 (10 μg/ml) and FAM-Ahx-FQIPYIIIK-NH_2 (10–100 μM) in DMEM enriched with $CaCl_2$ (50 μM) and $MgCl_2$ (50 μM) for 30 min at 37°C. D54 cells were incubated with: FAM-Ahx-RGD-NH_2 (10 μM), Rho-Ahx-RGD (10 μM), (10 μM), FAM-Ahx-FQSIYPpIK-NH_2 (10 μM) as well as (10 μM) Rho-Ahx-RGD-NH_2 (10 μM) and FAM-Ahx-FQSIYPpIK-NH_2 (10-100 μM) in DMEM enriched with $CaCl_2$ (50 μM) and $MgCl_2$ (50 μM) for 30 min at 37°C. Hereafter, the media was removed and the cells were washed, detached by scraping or by treatment with 1× pronase/EDTA for 15 min at 37°C, suspended in PBS enriched with $CaCl_2$ (50 μM) and $MgCl_2$ (50 μM), and analyzed by flow cytometry (Supplemental Experimental Procedures). Compensation was applied between the FAM/PerCP and the FAM/Rho fluorophore pairs.

Cytotoxicity Assays

D54 and HEK293T-CCR6 cells were incubated with FAM-labeled peptide (100 μM) in appropriate growth media for 24 hr at 37°C (Supplemental Experimental Procedures) and the media was removed and fresh media was added and the incubation continued for 24 hr. Cells were washed with PBS and MTT (50 μl, 1 mg/ml) in IMDM was added and incubated for 4 hr at 37°C, followed by addition of MTT solubilizing solution (Supplemental Experimental Procedures). Hereafter, the well-plate was shaken till complete dissolution of the formazan crystals and the absorbance was measured at 570 nm in a microplate reader (Supplemental Experimental Procedures). Untreated cells were considered to be 100% viable.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2011.07.017.

ACKNOWLEDGMENTS

The authors thank the EPSRC and BBSRC for funding.

Received: May 30, 2011
Revised: July 5, 2011
Accepted: July 6, 2011
Published: October 27, 2011

REFERENCES

Ben-Dor, A., Karp, R., Schwikowski, B., and Yakhini, Z. (2000). Universal DNA tag systems: a combinatorial design scheme. J. Comput. Biol. *7*, 503–519.

Brenner, S., and Lerner, R.A. (1992). Encoded combinatorial chemistry. Proc. Natl. Acad. Sci. USA *89*, 5381–5383.

Buller, F., Zhang, Y., Scheuermann, J., Schäfer, J., Bühlmann, P., and Neri, D. (2009). Discovery of TNF inhibitors from a DNA-encoded chemical library based on diels-alder cycloaddition. Chem. Biol. *16*, 1075–1086.

Clark, M.A., Acharya, R.A., Arico-Muendel, C.C., Belyanskaya, S.L., Benjamin, D.R., Carlson, N.R., Centrella, P.A., Chiu, C.H., Creaser, S.P., Cuozzo, J.W., et al. (2009). Design, synthesis and selection of DNA-encoded small-molecule libraries. Nat. Chem. Biol. *5*, 647–654.

Coughlan, L., Vallath, S., Saha, A., Flak, M., McNeish, I.A., Vassaux, G., Marshall, J.F., Hart, I.R., and Thomas, G.J. (2009). In vivo retargeting of adenovirus type 5 to alphavbeta6 integrin results in reduced hepatotoxicity and improved tumor uptake following systemic delivery. J. Virol. *83*, 6416–6428.

Debaene, F., Da Silva, J.A., Pianowski, Z., Duran, F.J., and Winssinger, N. (2007). Expanding the scope of PNA-encoded libraries: divergent synthesis of libraries targeting cysteine, serine and metallo-proteases as well as tyrosine phosphatases. Tetrahedron *63*, 6577–6586.

Demidov, V.V., Potaman, V.N., Frank-Kamenetskii, M.D., Egholm, M., Buchard, O., Sönnichsen, S.H., and Nielsen, P.E. (1994). Stability of peptide nucleic acids in human serum and cellular extracts. Biochem. Pharmacol. *48*, 1310–1313.

Díaz-Mochón, J.J., Bialy, L., and Bradley, M. (2006). Dual colour, microarray-based, analysis of 10,000 protease substrates. Chem. Commun. (Camb.) *38*, 3984–3986.

Díaz-Mochón, J.J., Bialy, L., Keinicke, L., and Bradley, M. (2005). Combinatorial libraries—from solution to 2D microarrays. Chem. Commun. (Camb.) *11*, 1384–1386.

Filmore, D. (2004). It's a GPCR world: cell-based screening assays and structural studies are fueling G-protein coupled receptors as one of the most popular classes of investigational drug targets. Mod. Drug Discov. *7*, 24–28.

Franch, T. Lundorf, M.D., Jacobsen, S.N., Olsen, E.K., Andersen, A.L., Holtmann, A., Hansen, A.H., Sorensen, A.M., Goldbech, A., de Leon, D., et al. June 2007. Enzymatic encoding methods for efficient synthesis of large libraries. U.S. patent WO2007062664.

Fueyo, J., Gomez-Manzano, C., Puduvalli, V.K., Martin-Duque, P., Perez-Soler, R., Levin, V.A., Yung, W.K., and Kyritsis, A.P. (1998). Adenovirus-mediated p16 transfer to glioma cells induces G1 arrest and protects from paclitaxel and topotecan: implications for therapy. Int. J. Oncol. *12*, 665–669.

Gartner, Z.J., Tse, B.N., Grubina, R., Doyon, J.B., Snyder, T.M., and Liu, D.R. (2004). DNA-templated organic synthesis and selection of a library of macrocycles. Science *305*, 1601–1605.

Gomez-Manzano, C., Fueyo, J., Kyritsis, A.P., Steck, P.A., Roth, J.A., McDonnell, T.J., Steck, K.D., Levin, V.A., and Yung, W.K. (1996). Adenovirus-mediated transfer of the p53 gene produces rapid and generalized death of human glioma cells via apoptosis. Cancer Res. *56*, 694–699.

Harris, J.L., and Winssinger, N. (2005). PNA encoding (PNA=peptide nucleic acid): from solution-based libraries to organized microarrays. Chemistry *11*, 6792–6801.

Insel, P.A., Tang, C.-M., Hahntow, I., and Michel, M.C. (2007). Impact of GPCRs in clinical medicine: monogenic diseases, genetic variants and drug targets. Biochim. Biophys. Acta *1768*, 994–1005.

Kuratomi, Y., Nomizu, M., Nielsen, P.K., Tanaka, K., Song, S.-Y., Kleinman, H.K., and Yamada, Y. (1999). Identification of metastasis-promoting sequences in the mouse laminin alpha 1 chain. Exp. Cell Res. *249*, 386–395.

Melkko, S., Scheuermann, J., Dumelin, C.E., and Neri, D. (2004). Encoded self-assembling chemical libraries. Nat. Biotechnol. *22*, 568–574.

Miller, W.H., Keenan, R.M., Willette, R.N., and Lark, M.W. (2000). Identification and in vivo efficacy of small-molecule antagonists of integrin alphavbeta3 (the vitronectin receptor). Drug Discov. Today *5*, 397–408.

Morgan, B., Hale, S., Arico-Muendel, C.C., Clark, M., Wagner, R., Kavarana, M.J., Creaser, S.P., Franklin, G.J., Centrella, P.A., Israel, D., et al. June 2005. Methods for synthesis of encoded libraries. U.S. patent WO2005058479.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods *65*, 55–63.

Needels, M.C., Jones, D.G., Tate, E.H., Heinkel, G.L., Kochersperger, L.M., Dower, W.J., Barrett, R.W., and Gallop, M.A. (1993). Generation and screening of an oligonucleotide-encoded synthetic peptide library. Proc. Natl. Acad. Sci. USA *90*, 10700–10704.

Nielsen, P.E. (2004). Peptide Nucleic Acids: Protocols and Applications, Second Edition (Wymondham, UK: Horizon Bioscience).

Nielsen, J., Brenner, S., and Janda, K.D. (1993). Synthetic methods for the implementation of encoded combinatorial chemistry. J. Am. Chem. Soc. *115*, 9812–9813.

Scheuermann, J., Dumelin, C.E., Melkko, S., and Neri, D. (2006). DNA-encoded chemical libraries. J. Biotechnol. *126*, 568–581.

Pianowski, Z.L., and Winssinger, N. (2008). Nucleic acid encoding to program self-assembly in chemical biology. Chem. Soc. Rev. *37*, 1330–1336.

Pouchain, D., Díaz-Mochón, J.J., Bialy, L., and Bradley, M. (2007). A 10,000 member PNA-encoded peptide library for profiling tyrosine kinases. ACS Chem. Biol. *2*, 810–818.

Svensen, N., Díaz-Mochón, J.J., and Bradley, M. (2008). Microwave-assisted orthogonal synthesis of PNA-peptide conjugates. Tetrahedron Lett. *49*, 6498–6500.

Svensen, N., Díaz-Mochón, J.J., and Bradley, M. (2011b). Encoded peptide libraries and the discovery of new cell binding ligands. Chem. Commun. (Camb.) *47*, 7638–7640.

Svensen, N., Díaz-Mochón, J.J., and Bradley, M. (2011c). Microarray generation of thousand-member oligonucleotide libraries. PLoS One, in press. 10.1371/journal.pone.0024906.

Svensen, N., Díaz-Mochón, J.J., Dhaliwal, K., Planonth, S., Dewar, M., Armstrong, J.D., and Bradley, M. (2011a). Screening of a combinatorial homing peptide library for selective cellular delivery. Angew Chem. Int. Ed. Engl. *50*, 6133–6136.

Urbina, H.D., Debaene, F., Jost, B., Bole-Feysot, C., Mason, D.E., Kuzmic, P., Harris, J.L., and Winssinger, N. (2006). Self-assembled small-molecule microarrays for protease screening and profiling. ChemBioChem *7*, 1790–1797.

Winssinger, N., Damoiseaux, R., Tully, D.C., Geierstanger, B.H., Burdick, K., and Harris, J.L. (2004). PNA-encoded protease substrate microarrays. Chem. Biol. *11*, 1351–1360.

Winssinger, N., Ficarro, S., Schultz, P.G., and Harris, J.L. (2002). Profiling protein function with small molecule microarrays. Proc. Natl. Acad. Sci. USA *99*, 11139–11144.

Winssinger, N., Harris, J.L., Backes, B.J., and Schultz, P.G. (2001). From split-pool libraries to spatially addressable microarrays and its application to functional proteomic profiling. Angew. Chem. Int. Ed. Engl. *40*, 3152–3155.

Wrenn, S.J., Weisinger, R.M., Halpin, D.R., and Harbury, P.B. (2007). Synthetic ligands discovered by in vitro selection. J. Am. Chem. Soc. *129*, 13137–13143.

Zaballos, A., Varona, R., Gutiérrez, J., Lind, P., and Márquez, G. (1996). Molecular cloning and RNA expression of two new human chemokine receptor-like genes. Biochem. Biophys. Res. Commun. *227*, 846–853.