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Journal of Immunological Methods 309 (2006) 120–129

JIM
Journal of Immunological Methods

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Research paper

Isolation of cell specific peptide ligands using fluorescent bacterial display libraries

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Received 3 May 2005; received in revised form 15 August 2005; accepted 28 November 2005
Available online 11 January 2006

Abstract

Methods for identifying and producing cell specific affinity reagents are critical in cell detection, separation, and therapeutic delivery applications, yet remain difficult and time consuming. To address these limitations, a rapid and quantitative screening approach was developed using intrinsically fluorescent bacterial display peptide libraries and fluorescence-activated cell sorting (FACS). High-throughput screening of fluorescent libraries yielded a panel of peptide ligands mediating specific recognition of human breast cancer tumor cells. Clonal populations of fluorescent, peptide-displaying bacteria enabled single-step, fluorescent labeling of the target cells for cytometry and microscopy analysis. Isolated peptides could be categorized into several distinct groups possessing strong consensus sequences with as many as six identities. Importantly, individual clones exhibited high specificity target cell binding, with more than 80-fold increased binding to tumor cells (ZR-75-1) relative to cell lines derived from healthy tissue (HMEC, MCF-10A). Fluorescent display libraries thus provide a powerful new methodology for parallel identification of cell specific affinity ligands.

Keywords: Bacterial display; Breast cancer; FACS; Peptide library; Targeting ligand

1. Introduction

Cell specific affinity reagents are widely used in biotechnology applications ranging from cell detection, separation, and characterization (Brown, 2000), to the delivery of imaging and therapeutic agents to specific cell types in vivo (Arap et al., 1998; Landon and Deutscher, 2003). Cell specific reagents have been discovered almost exclusively using antibody or peptide libraries displayed on filamentous bacteriophage (Brown, 2000; Landon and Deutscher, 2003). Phage display libraries have been applied in ligand selections using cultured cells, animals (Pasqualini and Ruoslahti, 1996), or human patients (Zurita et al., 2004), yielding peptides specific for cell surface markers (Rasmussen et al., 2002). The principle advantage of display library methodologies is that selection can be performed in a native-like, membrane-bound environment without a priori knowledge of the target cell receptors. Peptide ligands generated in this manner have proven useful for in vivo imaging studies (Ladner, 1999), therapeutic targeting (Park et al., 2002; Sapra and Allen, 2002), and for identification of cell specific surface markers (Pasqualini et al., 2000; Landon and Deutscher, 2003).

Abbreviations: FACS, fluorescence activated cell sorting; GFP, green fluorescent protein; OmpA, outer membrane protein A; CPX, circularly permuted outer membrane protein OmpX; SA-PE, streptavidin R-phycoerythrin

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doi:10.1016/j.jim.2005.11.021

The development of cell specific affinity reagents using display technology would benefit from more efficient and quantitative screening processes. Previous efforts to identify cell binding peptides using phage and cell display methodologies have relied upon a selection process referred to as panning (Lu et al., 1995; Brown et al., 2000; Nakajima et al., 2000). These studies have successfully enabled isolation of cell binding or invading peptides, though rarely reveal a consensus motif among the isolated peptides (Brown et al., 2000; Nakajima et al., 2000; Taschner et al., 2002). Methods for ligand selection using display on the outer surface of cells are attractive since fluorescence-activated cell sorting (FACS) instrumentation can be applied for quantitative clone screening and analysis (Feldhaus et al., 2003; Bessette et al., 2004). Importantly, cell display methods allow direct clone recovery from target cells and amplification by regrowth, without requiring separation or infection steps. Consequently, improved cell display library and screening methodologies would substantially simplify cell specific ligand isolation, as well as enable quantitative library screening and clone analysis.

A high-throughput, quantitative screening approach was developed to isolate cell specific affinity reagents using fluorescent bacterial display peptide libraries coupled with FACS. Fluorescent display libraries were constructed that co-express a green fluorescent protein (GFP) intracellularly, while efficiently displaying peptides on the surface of *Escherichia coli* to the extracellular environment. In this way, living bacterial cells served as fluorescent affinity reagents, thereby enabling quantitative library screening and subsequent clone characterization. To demonstrate the utility of this approach, two different fluorescent bacterial display libraries were screened for peptides binding to human breast cancer tumor cells. Quantitative screening using FACS succeeded in identifying, for the first time, an array of unique peptide consensus groups binding to the target cells. By incorporating negative selections using healthy breast derived cell lines, highly specific tumor cell binding peptides were identified.

2. Materials and methods

2.1. Cell lines, vectors, and reagents

The human breast ductal carcinoma cell line (ZR-75-1) and the immortalized human mammary cell line (MCF-10A) were obtained from ATCC (American Type Culture Collection, Manassas, VA). Clonetics® human mammary epithelial cells (HMEC) were from Cambrex Bio Science (Walkersville, MD). ZR-75-1 tumor cells were cultured in RPMI-1640 medium (ATCC) with 10% fetal bovine serum (Invitrogen, Carlsbad, California). Normal mammary cells were cultured in complete mammary epithelial cell medium (MEGM, Cambrex Bio Science). The OmpA 15mer bacterial display library was propagated as previously described (Bessette et al., 2004). For the specificity selection, two random peptide libraries of the form X_{15} and $X_2CX_7CX_2$ were constructed as fusions to the extracellular N-terminus of a circularly permuted variant of outer membrane protein OmpX (CPX) (Rice et al., in press). Following the native signaling sequence of OmpX, CPX was constructed by fusion of the native C- and N-terminus of OmpX with a GGSG linker, and by opening loop 2 between residues S53 and S54 yielding a scaffold with extracellular C- and N-termini. Each library was comprised of 2×10^9 members. The gene encoding the CPX scaffold was inserted into the multiple cloning site of pBAD33 (Guzman et al., 1995) downstream of alajGFP1 (Bessette and Daugherty, 2004). This bicistronic cassette allowed for simultaneous expression of both alajGFP1 and CPX under the control of the arabinose inducible *araBAD* promoter (Guzman et al., 1995).

2.2. Library selection, screening, and analysis

For the first two cycles of selection, bacteria binding to tumor cells were selected using co-sedimentation. Frozen aliquots of 2.5×10^{11} cells containing the OmpA surface display library were thawed and grown overnight in Luria Bertani (LB) broth supplemented with 34 μg/mL chloramphenicol (Cm) and 0.2% D-(+)-glucose. The bacteria were subcultured 1:50 for 3 h in LB supplemented with Cm and induced with 0.02% L-(+)-arabinose for 2 h. Tumor cells were harvested from dishes, resuspended in conical tubes, and co-incubated with 100-fold excess bacterial cells for 1 h on an inversion shaker at 37 °C. For round 1, 10^8 tumor cells were incubated with 10^{10} bacterial cells, while in round 2, 10-fold fewer cells of each type were used. Cell suspensions were centrifuged at 2500 rpm for 4 min and non-binding bacteria in the supernatant were removed. Tumor cells were washed three times in phosphate buffered saline (PBS) and resuspended in LB for overnight growth of binding bacteria. Recovered bacterial clones were counted by plating on LB-agar plates supplemented with Cm. A depletion step was performed between rounds one and two to remove any bacteria sedimenting as a result of increased aggregation by centrifuging bacteria at 2500 rpm for 4 min and recovering 95% of the supernatant.

For flow cytometric sorting and screening, plasmids of the unselected and selected OmpA library populations were extracted from bacterial cells using Qiagen Miniprep kits (Qiagen, Valencia, CA). Subsequently, these plasmids were transformed into *E. coli* MC1061/pUC18-EGFP for constitutive GFP expression. Sample preparation for FACS was performed using similar techniques as described above with 10^6 tumor cells suspended in 2 mL of culture medium. After coincubation with 100-fold excess bacteria and washing, tumor cells were resuspended in a final volume of 3 mL of PBS and incubated on ice. The round two population was analyzed immediately by flow cytometry using a Partec PAS III (Münster, Germany) equipped with a 100 mW Argon (488 nm) laser, and fluorescent tumor cells were recovered by sorting to yield the round three population. Library enrichment was assayed by flow cytometry after coincubation of 10^6 tumors cells with 50-fold excess bacteria and performing two washes with PBS. The percentage of tumor cells with bacteria bound and the average fluorescence intensity over background were determined by flow cytometric analysis of 2×10^4 tumor cells. Tumor cell binding was verified for individual clones by flow cytometry, and peptide sequences were determined via DNA sequencing. Sequence alignment was performed with AlignX® (Invitrogen) using a modified ClustalW algorithm. The probability associated with finding amino acid identities and similarities between two sequences in the same position was performed by calculating the probability of each codon (NNS), and then multiplying the individual positions together for an overall probability. For alignment, amino acid similarity was defined as follows: A/G/V/I/L/M; F/Y/W/M; S/T; D/E; N/Q; R/H/K.

To identify tumor specific peptides using the CPX display libraries, frozen aliquots of the 15mer and constrained 7mer libraries were grown as described above. Libraries were pooled together, and one round of cosedimentation was performed as above with bacteria and tumor cells coincubated in PBS. Prior to the second round, a negative selection was performed by panning 5×10^7 bacteria against 10^7 adhered normal cells containing a mixture of MCF-10A and HMEC cells. Bacteria not bound to normal cells in the supernatant were removed and immediately incubated with 10^7 suspended ZR-75-1 cells for a second round of positive selection using cosedimentation. Following an additional round of similar negative panning and positive coincubation with 10-fold fewer cells, tumor cells with fluorescent bacteria bound were immediately sorted using a FACSAria (Becton Dickinson, San Jose, CA).

2.3. Soluble peptide interactions

Two cyclic peptides with *N*-terminal biotin (bio) and disulfide bond formation (pep5: (bio)GGCLQILPTLSECFGR and pep17 (bio)GLKVCGRYPGICDGIR) and a linear peptide binding to human erythrocytes (unpublished) pepE1: (bio)GKYTWYGYSLRANWMR) synthesized by Invitrogen (Carlsbad, CA) were used for tumor cell binding studies. Peptides were purified using reverse phase HPLC on a C18 column and verified using MALDI-TOF mass spectrometry. Peptides were dissolved in PBS and incubated with 5×10^5 tumor cells at a final concentration of 100 μM for 1 h. Cells were washed three times at 4 °C with 10-fold excess PBS and labeled with 50 nM streptavidin R-phycoerythrin (SA-PE). Red fluorescence (610 emission filter) was measured by flow cytometry (Partec PAS-III) with 488 nm excitation.

2.4. Specificity assay of isolated clones

To characterize specificity, individual clones from the CPX library screen were analyzed using cytometry for binding toward ZR-75-1, MCF-10A, and HMEC cell lines. For binding assays, 10^6 mammalian cells were scanned on a FACSAria after a coincubation with 5×10^7 bacteria for 1 h followed by two washes.

2.5. Fluorescence microscopy

One day prior to analysis, tumor cells (5×10^5) were seeded in culture dishes for microscopy analysis. Bacteria overexpressing CPX (MC1061/pBAD33-GFP-CPX), clone 20 (MC1061/pBAD33-GFP-CPX-20), or clone 23 (MC1061/pBAD33-GFP-CPX-23) were coincubated at 37 °C for 45 min in PBS and washed four times. Adherent cells were photographed using a Zeiss Axiovert 25 microscope with a CoolSnap HQ camera (Roper Scientific) with an epi-fluorescence attachment and GFP filter cube. Fluorescence and bright-field images were recorded and overlaid at 1000× magnification.

3. Results

3.1. Bacterial display library screening

To identify cell specific peptide ligands, two different approaches were used. In the first approach, a large bacterial display peptide library composed of more than 10^{10} unique, random 15mer peptides displayed as insertional fusions within the first extracellular loop of

outer membrane protein OmpA (Bessette et al., 2004) was used for selection and screening. Peptide sequences mediating binding to the target cells, human invasive ductal carcinoma cell line ZR-75-1, were isolated using a two-step process. First, non-fluorescent bacteria bound to tumor cells were enriched by cosedimentation to reduce library diversity. The resulting library plasmids were transformed into bacteria that constitutively express EGFP, and this library was screened for tumor cell binding using FACS (Fig. 1). Bacterial library populations resulting from each cycle of selection and screening were analyzed by flow cytometry to track enrichment, and compared using two different metrics: (i) the percentage of tumor cells with one or more bound bacteria and (ii) the mean fluorescence of the tumor cell population, indicating the number of bacteria bound per cell (Fig. 1G). Binding was strongly dependent upon cell concentrations and the ratio of bacteria to tumor cells (data not shown). Thus, flow cytometry provided quantitative measurements that enabled direct assessment of the number of library clones binding to the target cells as well as individual clone function.

Using FACS as a high-throughput screening tool, the bacterial library was screened for cell binding peptides. Tumor cells exhibited 10–100-fold increased green fluorescence when fluorescent bacteria were bound, allowing for simple discrimination from unlabeled

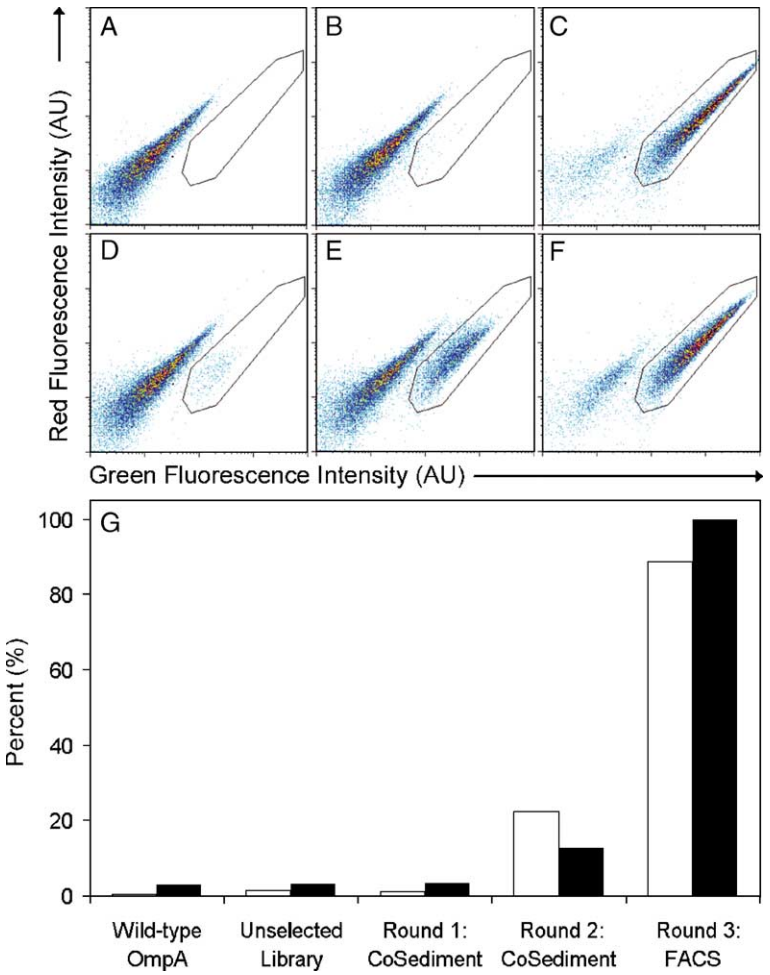

Fig. 1. OmpA library analysis and screening using FACS. Two color flow cytometric plots allow for simple discrimination between the autofluorescence of ZR-75-1 cells and subsequent increase in green fluorescence due to binding of GFP-expressing bacteria. Fluorescence of (A) ZR-75-1 tumor cells alone, and (B) ZR-75-1 after incubation with bacterial cells expressing wild-type OmpA; or (C) clone 2. The library population (D) prior to sorting, (E) after two rounds of co-sedimentation; (F) after two rounds of co-sedimentation and one round of FACS. (G) Enrichment of clones binding to ZR-75-1 cells as measured by the percentage of tumor cells with one or more bacteria bound (white bars, based on gate in A), and the normalized mean green fluorescence of each population (black).

tumor cells (Fig. 1A). Non-specifically bound bacteria, displaying the carrier OmpA alone, were effectively removed by washing (Fig. 1B). In contrast, clone 2 (Table 1) which was 10-fold more specific for tumor cells versus normal cells (data not shown), efficiently labeled 90% of tumor cells (Fig. 1C). Prior to library screening, the frequency of tumor cell binding library clones was measured to be roughly 1% using flow cytometry (Fig. 1D,G). To facilitate library screening via FACS, the full library diversity (10^{10}) was reduced to $\sim 10^{7}$ unique clones by recovering bacteria that cosediment with tumor cells (Fig. 1E,G). The reduced library was subsequently screened for tumor cell binding by sorting tumor cells with one or more bound bacteria (Fig. 1F, Table 1) to isolate library clones binding to both rare and abundant cell surface receptors on all tumor cells.

Table 1
Consensus motifs of ZR-75-1 binding peptides selected using the OmpA library

Clone	Frequency[a]	Sequence	Fold increase[b]
1	1	VCRLMRGRCLLYSVF	15
2	2	TCVLHRQRCLMFTLR	99
3	1	ICVNIKKSLWACEIR	13
4	1	NCVRILMTFLDCTID	33
5	3	GCLQILPTLSECFGR	32
6	1	KQRGATMVLRTYTLR	17
7	1	YERRPTLVLRTWRPW	178
8	3	SVLVWVKDRGWRPAR	33
9	1	LYAHYDESRGWRWIR	176
10	1	WKFVWIINSRLREQA-	3
11	1	WARVLLIEGRLIVCE-	46
12	1	-GNVLGKDYRLVKHVN	148
13	1	ARWIWYRNTATLNSV-	48
14	2	-CWILPYNTRTRCPLR	46
15	2	NQGLIGECHAYWCHG--	26
16	1	-NLIIGFCWLKKCP-IR	44
17	1	--LKVCGRYPGICDGIR	87
18	3	-WWDMVSDRYIWKPVK	30
19	1	TQWIIPSKLAIKTPS-	45

[a]Frequency of occurrence among binding clones in the round 3 population.
[b]Fold-increase in binding to ZR-75-1 cells calculated by comparing percentage of cells in Fig. 1A gate to that of cells expressing OmpA alone.

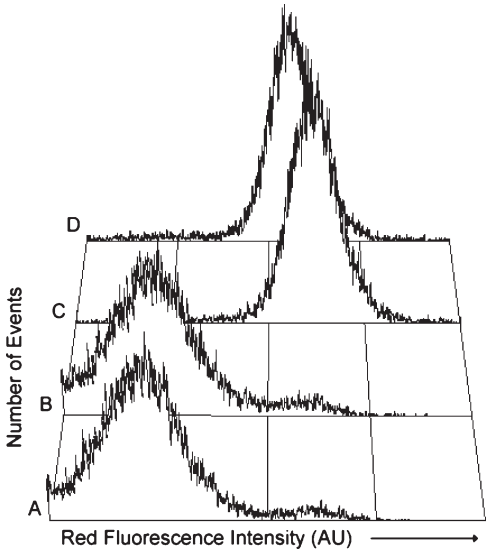

Fig. 2. Soluble peptide recognition of target cells. Binding of fluorescently conjugated, soluble peptides to ZR-75-1 as measured using flow cytometry. ZR-75-1 cells were incubated in buffer (A) without peptide, or with biotinylated peptides, (B) erythrocyte binding peptide (pepE1), (C) pep5, and (D) pep17, and then labeled with SA-PE.

3.2. Characterization of tumor binding peptides isolated from the OmpA library

The intrinsic fluorescence of the co-transformed OmpA bacterial display library enabled rapid characterization using flow cytometry. Of 70 isolated clones, 48 contained unique sequences. After screening, 36 of 48 of these exhibited binding to tumor cells as measured using flow cytometry. Within these 36 binding clones, phylogenetic analysis (Methods) identified 19 clones sharing differing degrees of similarity (Table 1). Individual sequences shared as many as six identities and four similarities (clones 1 and 2, Table 1). Five peptides in two different consensus groups (CxLxRxRCL and CVxILxTLx$^{D}/_{E}$CxIR) possessed cysteine residues at identical positions (Table 1).

To determine whether the peptides identified would retain binding function in the absence of the OmpA display scaffold, soluble peptides corresponding to two arbitrarily selected clones (5 and 17) that exhibited different extent of tumor cell binding were synthesized and conjugated to biotin at their *N*-termini (pep5 and pep17). Less than 1.5% of ZR-75-1 cells incubated in buffer without peptide were labeled nonspecifically with SA-PE as measured using flow cytometry (Fig. 2A). An unrelated erythrocyte binding peptide (pepE1) (unpublished data) also exhibited background fluorescence (Fig. 2B). In contrast, both of the isolated tumor

binding peptides exhibited uniform binding to ZR-75-1 cells; pep5 under these conditions was 21-fold more fluorescent while pep17 showed 15-fold enhancement (Fig. 2C and D). These results demonstrate that peptide sequences identified using bacterial display loop insertions can retain cell binding function in the absence of the display scaffold in micromolar concentration ranges.

Isolated bacterial clones differed in their extent and specificity of tumor cell binding as measured using flow cytometry (Table 1). A subset of isolated clones (2, 3, 11, and 18) exhibited approximately 10-fold enhanced tumor cell binding specificity as measured by fluorimetry (data not shown), suggesting that these clones recognize receptors overexpressed on tumor cells. However, since negative selections with non-target cell lines were not used, many isolated clones were not specific for tumor cells (data not shown), and also bound to two cell lines derived from healthy breast tissues (MCF-10A, HMEC). One drawback observed using this two plasmid expression system was that constitutive expression of EGFP from a high-copy plasmid resulted in a large standard deviation in the whole cell fluorescence intensity for individual library members. While this problem did not prevent the isolation of binding clones, fluorescence variations would be expected to result in some loss of diversity, as well as to reduce the accuracy in ranking of individual isolated clones using clone fluorescence.

3.3. Selection of ZR-75-1 specific peptides using CPX display libraries

Given the modest specificity of clones identified using the OmpA library, we sought to develop an improved expression vector that would yield stable levels of GFP expression, and potentially yield peptides that are scaffold independent. Furthermore, we sought to increase the stringency of selection for specificity by performing negative selections against non-target cell lines. Thus, a bicistronic vector was constructed to enable simultaneous expression of a GFP well suited for FACS (Bessette and Daugherty, 2004) and an outer member protein scaffold enabling peptide display as N-terminal fusions rather than as insertions. In this display vector, peptides are genetically fused to the extracellular N-terminus of a circularly permuted variant of outer membrane protein OmpX (Rice et al., in press). Two peptide libraries of the form X_{15} and $X_2CX_7CX_2$ were pooled, and alternating positive and negative selections were performed on tumor and normal breast cells. Bacterial clones exhibiting specific tumor cell binding were enriched using two cycles of selection and then screened using FACS for strong binding to the target cells. Enrichment occurred at a rate comparable to that observed using the OmpA display library (data not shown).

3.4. Characterization of tumor binding peptides from the CPX libraries

From the selection and screening using CPX libraries, 8 of 20 individual isolated clones exhibited tumor cell binding and seven unique clones were identified (Table 2). Interestingly, 6 of 7 clones were derived from the 7mer library, and clone 22 (Table 2) from the 15mer library also contained two cysteines separated by eight residues thus revealing a strong preference for a disulfide constraint (Bessette et al., 2004). GFP expression levels did not vary substantially amongst these clones (data not shown). Given the relatively small number of clones isolated and characterized, strong consensus motifs were not observed. In addition to high levels of tumor cell binding, binding assays against normal breast cells revealed that clones 20–26 were highly specific for ZR-75-1 (Fig. 3). Clone 23 proved to be the most specific with 85-fold enhanced specificity when compared to the normal cell binding (Fig. 3). These results demonstrate that peptides isolated from bacterial display libraries yield highly specific clones when stringent selection and screening conditions are used.

The binding of fluorescent, peptide displaying bacteria (clones 20 and 23, Table 2) to the tumor cells was further characterized using fluorescence microscopy (Fig. 4). Bacteria overexpressing CPX alone were readily washed from tumor cells (Fig. 4A). In contrast, clones 20 and 23 bound efficiently to tumor cells, resulting in concentration dependent fluorescent

Table 2
Tumor cell (ZR-75-1) specific binding peptides identified using CPX bacterial display

Clone	Freq [a]	Sequence	Fold increase [b]
20	1	VPCQKRPGWVCLW	72
21	1	KWCVIWSKEGCLF	57
22 [c]	2	SSWCMRGQYNKICMW	12
23	1	VECYLIRDNLCIY	84
24	1	WWCLGERVVRCAH	67
25	1	FYCVIERLGVCLY	85
26	1	RVCFLWQDGRCVF	85

[a] Frequency of occurrence among binding clones from round 3.
[b] Fold increase in clone binding relative to cells that do not display a peptide.
[c] Isolated from the X_{15} library.

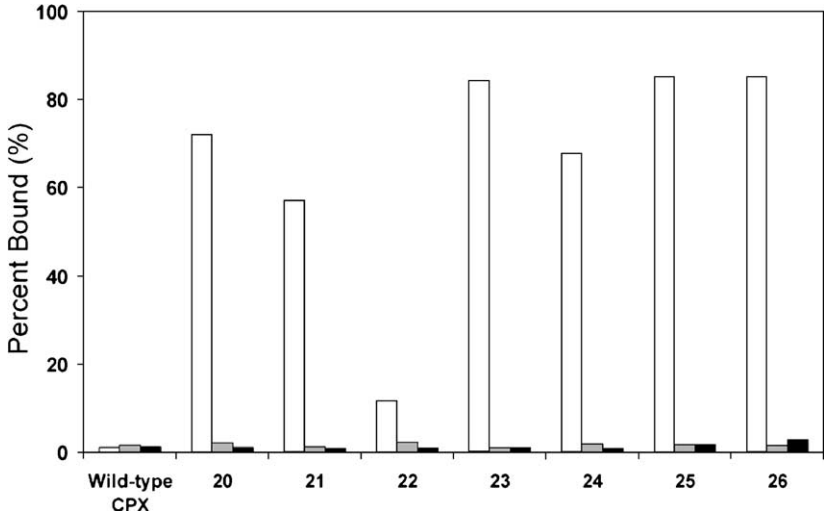

Fig. 3. Specificity of clones isolated from CPX display libraries. The percentage of mammalian cells with bound fluorescent bacteria after coincubation of tumor cells and a 50-fold excess of bacteria, as measured using flow cytometry (representative data). Binding of CPX specific clones to ZR-75-1 tumor cells (white bars) relative to normal cells lines, HMEC (grey) and MCF-10A (black).

labeling of tumor cells (Fig. 4B and C). Binding was apparently strong, since bacteria remained adherent after stringent washing. Bound bacteria were observed at discrete locations on the tumor cell surface (Fig. 4B and C), suggesting that bacterial cells were dividing at the cell surface leaving progeny co-localized, or that the cognate receptor is non-homogeneously distributed throughout the membrane. In some cases, bacterial localization of binding clones coincided with focal attachments. During prolonged incubation of bacteria and tumor cells, cell bound bacteria continued to grow on the cell surface (data not shown). These results indicate that whole, fluorescent bacteria displaying tumor-specific peptides can serve as self-renewing affinity reagents.

4. Discussion

Improved methodologies for identifying cell specific ligands are of substantial interest in biotechnology and medicine (Ruoslahti, 2000; Landon and Deutscher, 2003). With this aim, a simple and robust method was developed to identify cell specific binding peptides using large random peptide libraries displayed on fluorescent bacteria. This approach enabled quantitative library analysis and screening using FACS, and resulted in the identification of a panel of tumor cell specific binding ligands. Several unique consensus groups were identified from the large group of peptides isolated using the OmpA scaffold, providing a "receptor specificity fingerprint" differing from that of cells derived from normal breast tissue. The screening of bacterial libraries is simplified owing to straightforward manipulation of bacterial cells, the absence of elution and infection steps, tight regulation of peptide display, the ability to use intracellular fluorescent or luminescent markers, and the use of FACS for quantitative library screening and clone analysis without the use of secondary labels.

Fluorescent display libraries enabled quantitative screening via FACS, as an alternative to conventional panning or co-sedimentation (Taschner et al., 2002). Additionally, this approach enabled direct analysis of isolated clones using flow cytometry, fluorimetry, and fluorescence microscopy. Relatively few studies using phage display have attempted to harness fluorescent phage for clone analysis and screening by labeling phage with fluorophores, either covalently or indirectly. For example, phage particles have been labeled with fluorescent probes recently by a covalent linkage of pVIII phage coat proteins with amine-reactive fluorochromes (Jaye et al., 2004). Alternatively, the screening of a phage display antibody library for antibodies that recognize dendritic cells was accomplished using a more complicated tertiary labeling scheme, wherein cell-bound phage were labeled with anti-phage sheep antibodies followed by PE-conjugated donkey anti-sheep antibodies (Lekkerkerker and Logtenberg, 1999). In the analysis of integrin-targeted phage, a chimeric phage display system was developed to display both a cell-targeting ligand and a streptavidin binding peptide on two different bacteriophage coat proteins.

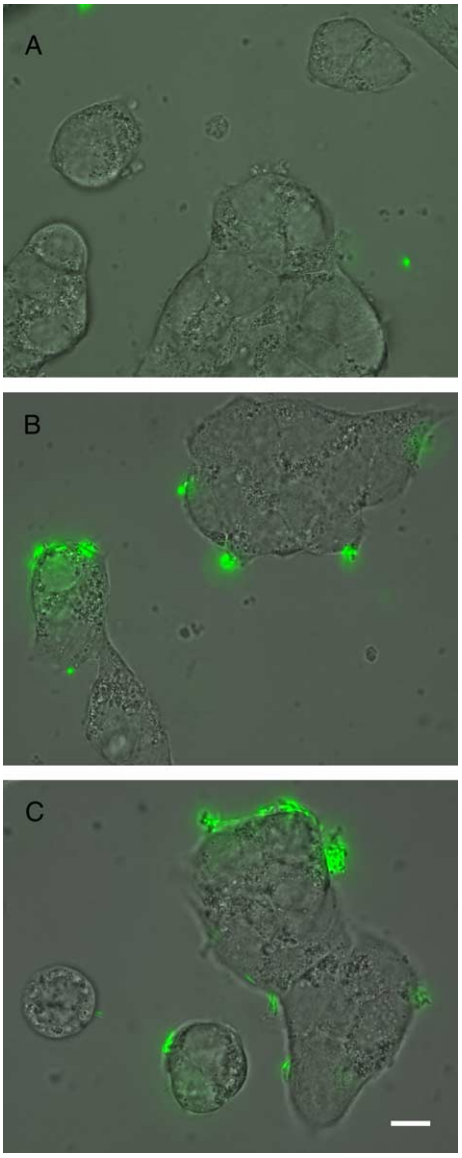

Fig. 4. Fluorescence imaging of cell binding clones from CPX display libraries. Representative fluorescence micrographs of bacteria binding to breast tumor cells (ZR-75-1) for cells (A) overexpressing CPX, (B) displaying clone 20, and (C) displaying clone 23 after a 45-min coincubation and four washes. Scale bar is 10 μm.

Subsequently, phage could be labeled with streptavidin-conjugated fluorophores, quantum dots, or magnetic beads enabling dynamic imaging, targeting, and separation (Chen et al., 2004). As an alternative to externally labeled phage, both phage and bacteria have been used as gene delivery vectors wherein receptor-mediated internalization can enable delivery of a protein or gene marker such as GFP (Larocca et al., 2002; Critchley et al., 2004). The fluorescent bacterial library methodology presented here provides a complementary approach enabling direct selection and quantitative screening for cell specific binding and invasion.

The absence of secondary labeling steps in fluorescent bacterial display may enable isolation of peptides binding to low density or unique tumor cell receptors since washing steps during labeling are eliminated. Since the binding of a single bacterium to a target cell is sufficient for detection and sorting, this approach should enable identification of ligands binding to low concentration, yet highly specific receptors. Such ligands would be especially useful for developing more accurate cell-type detection and identification assays and in therapeutic targeting applications that rely upon specificity for increased efficacy (Allen, 2002). In addition, the binding of fluorescent bacteria can be directly imaged to investigate receptor expression in different cell types, as well as density and localization. Finally, individual isolated clones may prove useful as affinity reagents for the identification and purification of the targeted receptors.

The emergence of several consensus motifs is unprecedented in cell-specific ligand isolation experiments, and suggests that the present methodology identified ligands recognizing many different cell surface receptors or epitopes. Most previous studies using peptide display libraries panned on whole cells have typically yielded either mixtures of clones with weak or no consensus sequence (Brown et al., 2000; Nakajima et al., 2000; Takahashi et al., 2003), or one dominant clone (Spear et al., 2001; Oyama et al., 2003), and rarely yield multiple consensus sequences in a single selection experiment (Muller et al., 2003). The fact that several peptide consensus groups were apparent amongst the sequences isolated using fluorescent bacterial display suggests that these clones recognize different cell surface receptors and that this methodology is not prone to undesired clonal biases that arise during some library selection protocols (Daugherty et al., 1999). Two of the peptides identified using an insertional fusion library in this study retained cell binding function in soluble form and exhibited binding at micromolar concentrations, comparable to those of peptides isolated using phage display (Landon and Deutscher, 2003). Given that display level and washing stringency influence the strength of interaction, improved binding affinities could likely be obtained using reduced display levels or by grafting peptides into constrained, soluble scaffolds (Bessette et al., 2004).

Peptides that mediate highly specific target cell binding were identified in this study using negative selections, or library depletion, with cell lines derived from healthy breast tissue. Remarkably, this stringent

selection scheme yielded one clone exhibiting more than 80-fold specificity for tumor cells versus two cell lines derived from healthy breast tissue. In fact, these specific clones did not bind to the non-target cells. This result suggests that these cell-specific peptides likely target unique, rather than overexpressed, receptors present on the target tumor cells. In comparison, screening without depletion yielded a maximum specificity of roughly 10-fold. Considering the variation in specificity among clones isolated without 'negative selection', depletion steps were clearly effective for removing non-specific clones. Thus, our results further illustrate that for in vitro selections, the identification of appropriate non-target cell lines is of critical importance. One of the principle advantages of fluorescent bacterial display is that both library pools and individual clones can be characterized quantitatively for binding using FACS. Given this feature, we anticipate that this methodology will be especially useful for large-scale identification of cell specific ligands and their cognate receptors, and subsequently to characterize and manipulate precisely defined cell populations.

Acknowledgments

We thank Chinmay Pangarkar and Samir Mitragotri for assistance with microscopy, and David Low and Annalee Nguyen for critically reading the manuscript. This work was supported in part by the National Institutes of Health-National Cancer Institute grant R21-CA107481 and University of California-Cancer Research Coordinating Committee grant CRCC 02-03.

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