A high-throughput alphavirus-based expression cloning system for mammalian cells

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We have developed a widely applicable functional genomics strategy based on alphavirus expression vectors. The technology allows for rapid identification of genes encoding a functional activity such as binding of a defined ligand. Complementary DNA (cDNA) libraries were expressed in mammalian cells following infection with recombinant Sindbis virus (SIN replicon particles), a member of the *Alphavirus* genus. Virus-infected cells that specifically bound a ligand of choice were isolated using fluorescence-activated cell sorting (FACS). Replication-competent, infective SIN replicon particles harboring the corresponding cDNA were amplified in a next step. Within one round of selection, viral clones encoding proteins recognized by monoclonal antibodies or Fc-fusion molecules could be isolated and sequenced. Moreover, using the same viral libraries, a plaque-lift assay was established that allowed the identification of secreted, intracellular, and membrane proteins.

Historically, knowledge of the function and therapeutic potential of a protein preceded identification and cloning of its corresponding gene. Today, high-throughput screening and sequencing have triggered an explosive growth in the identification of new genes[1]. However, while gene discovery has accelerated, the understanding of function has not kept pace. There exists today a large gap between sequence and functional information. The missing component between sequence data and identification of potential biopharmaceutical targets is a technology that can sift through the unworkably large pool of messenger RNAs (mRNAs) and can isolate, on the basis of a set of defined assay formats, the smallest possible subset of genes and proteins for further study. Here we describe an expression cloning system called DELphi (discovery of localized proteins) that may provide such a technology.

Expression cloning of cell surface molecules for eukaryotic cDNAs was first described more than a decade ago and was based on transient expression of cDNA libraries in COS cells[2–4]. However, inefficient transfection of the simian virus (SV40)-based expression plasmid libraries in many cell lines remained a limiting factor. In contrast, retrovirus-based expression allows infection of a variety of cell lines and even primary cells[5,6] and, with the advent of lentiviral vectors, possibly even of nondividing cells[7]. However, a problem with most retroviral vectors used for expression cloning is that they are replication-defective, making the recovery of the gene of interest sometimes difficult. One solution to this is to screen with replication-competent viruses such as baculoviruses[8]. However, these methods have the drawback of relying for library generation on homologous recombination, which impairs their diversity. Recombinant adenoviruses offer an advantage in this respect, because they permit libraries to be generated with conventional plasmid cloning technologies[9]. To date, RNA-based expression systems have not been routinely used for functional genomics.

Sindbis virus, of the *Alphavirus* genus, is a single-stranded RNA virus with positive polarity that offers numerous potential advantages over other expression systems used for functional genomics. The virus efficiently infects a wide range of host cells, including kidney cells, neuronal cells, muscle cells, liver cells, fibroblasts, and dendritic cells[10–15]. It induces high levels of expression of virally encoded genetic information shortly after infection[10–15]. RNA replication, as well as the entire virus life cycle, occurs in the cytoplasm. Therefore, expression levels are not dependent on integration sites or cell division. In addition, the viral genome is small and can easily be manipulated[11]. Expression vectors based on the full-length cDNA of the Sindbis genome with a size of only 11.7 kilobases[10,16,17] are available.

Here we have developed an expression screening technology based on alphaviral vectors. We demonstrate that the method is able to rapidly identify potential binding partners for cell surface, intracellular, and secreted proteins.

Results and discussion

Expression of one cDNA per cell using Sindbis virus. In order to generate positive controls for our screening system, cDNAs encoding green fluorescent protein (GFP), influenza virus hemagglutinin (HA), cluster of differentiation 40 (CD40), interleukin 13 (IL-13) receptor α-chain, C-X-C receptor 3 (CXCR3), and tumor necrosis factor (TNF) were cloned into an expression plasmid, referred to as pSinRepLIB (ref. 18; Fig. 1A), encoding the SIN virus–derived replicase. Heterologous cDNAs were inserted into pSinRepLIB under the control of the viral subgenomic promoter, which is recognized by the viral RNA-dependent RNA polymerase. Capped and polyadenylated RNA was generated by *in vitro* transcription of pSinRepLIB. Furthermore, RNA was transcribed from a helper plasmid (pDH-EB) encoding viral structural proteins[19]. Co-electroporation of both RNAs leads to the generation of replication-competent bipartite virus particles containing two strands of RNA (ref. 19), referred to as SIN replicon particles (Fig. 1A). Both RNA species contain viral packaging sequences to ensure efficient incorporation into viral particles.

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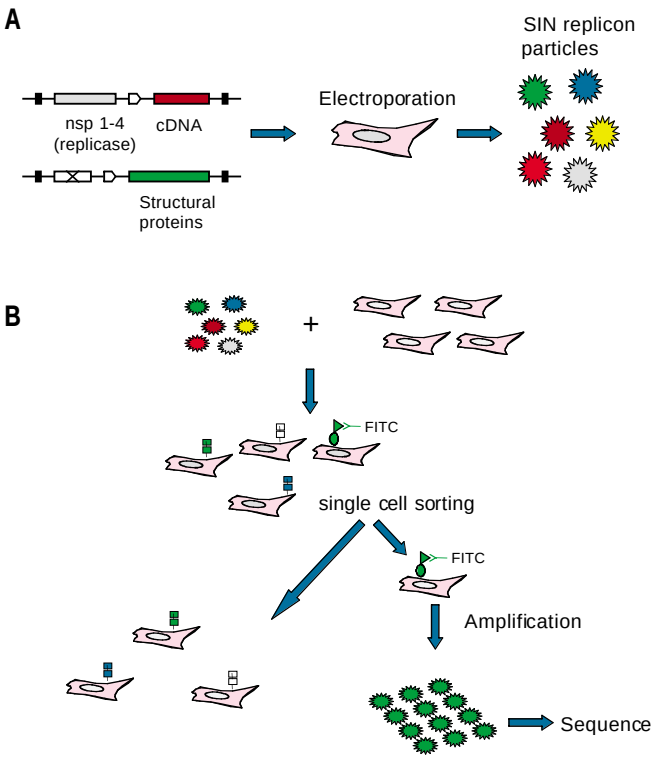

Figure 1. Scheme of the SIN replicon vectors and the expression screening strategy. (A) The pSinRepLIB vector encodes for the four Sindbis viral replicase proteins (nsp 1–4), a junction region, the viral subgenomic promoter (sp), and the multiple cloning site for cDNA integration. The viral structural genes and a packaging signal are encoded on a second plasmid referred to as pDH-EB. After an *in vitro* transcription step, the two RNA species are co-electroporated into cells, resulting in the generation of bipartite particles. (B) Diagrammatic representation of the DELphi isolation strategy based on the combination of the SIN replicon cDNA expression libraries and the FACS method.

An important factor for all known expression cloning systems is the difficulty of introducing genetic information into mammalian host cells at high efficiency and high expression levels with one cDNA per cell[20]. Single recombinant Sindis virus particles have been shown to be sufficient to induce a full replication cycle in infected cells leading to high-level expression of the recombinant gene in a variety of mammalian and insect cells[10]. To ensure that this was also the case using our SIN replicon system, we infected BHK cells at various multiplicities of infection (MOI) with SIN replicon particles encoding GFP. GFP expression was assessed by flow cytometry 6 h after infection (Fig. 2). At an MOI of 1, roughly 91% of the cells expressed GFP; at an MOI of 0.2, 22%; at an MOI of 0.1, 9.8%; and at an MOI of 0.05, 5.1% of the cells expressed GFP. These results suggested that infection occurred as a single-hit event. This indicated that a single viral particle was sufficient to initiate an infectious cycle. To further substantiate this, BHK cells were simultaneously infected with three types of recombinant SIN replicon particles encoding either the seven transmembrane receptor CXCR3, CD40, or HA with an MOI of approximately 0.02–0.08 each. After 7 h incubation at 37°C, samples of infected cells were stained for the expression of CXCR3, CD40, and HA and submitted to FACS (Fig. 2B). From these single sorted cells, virus was amplified, RNA isolated, cDNA generated, and the presence of DNA encoding CXCR3, CD40, and HA was assessed by PCR (Fig. 2C). The results demonstrate that cells expressing CXCR3, CD40, or HA exclusively contained the respective genes and cells were not multiply infected. Thus, the DELphi technology allows a controlled expression of only one cDNA library–derived gene per cell.

Isolation of infected ligand binding cells using a cell sorter. Next, we determined the sensitivity of our FACS-based screening strategy as outlined in Figure 1B. BHK cells infected with recombinant SIN replicon particles harboring cDNA of human α1-chain of IL-13 receptor were diluted with BHK cells infected with control SIN replicon particles. The dilutions ranged from 1:1,000 up to 1:500,000 cells (Fig. 3). Infected cells were stained by indirect immunofluorescence using human flag-tagged IL-13, and single cells positive for IL-13 binding were sorted by FACS (Fig. 3) into 24-well plates containing BHK cells. Upon virus spread in individual wells, populations of cells expressing IL-13 receptor could be visualized by indirect immunostaining 48 h later (Fig. 3). Similar results were obtained if viruses rather than infected cells were mixed (data not shown). Thus, the DELphi technology allowed single-step recovery of genes present at frequencies as low as 1:500,000, thereby increasing the chance of cloning low-abundance cDNAs. By making multiple rounds of selection, this efficiency could potentially be increased.

Generation of libraries. To test for the ability of our screening system to detect genes from cDNA libraries, we cloned cDNAs derived from human nonstimulated peripheral blood monocytes, human spleen, mouse spleen, and mouse brain into pSinRepLIB, and generated viral libraries (see one representative example in Table 1). An average of 40% of the genes were full-length and the number of primary clones was generally $>10^6$. Although the average size of inserts in the viral library was smaller than in the cDNA library, the overall quality was still good (Table 1), and inserts with sizes up to 4 kilobases could be identified (data not shown). Furthermore, the proportion of viruses carrying inserts with sizes larger than 2 kilobases was markedly increased by using size-fractionated libraries (data not shown). Additionally, we were able to recover rare genes such as *Mcp-1*, *Sdf1*, and *Mig* from the viral mouse brain library.

Cloning of receptors from libraries. We then used our screening assay to isolate antigens from the viral libraries using monoclonal antibodies. BHK cells were infected with SIN replicon particles encoding cDNA from a mouse spleen. The infected cells were incubated with the monoclonal antibody E53 (C. Ruedl, unpublished data), which specifically recognizes $CD8^+$ dendritic cells and $CD8^+$ T cells (Fig. 4A), although the molecular target of E53 is unknown. Single cells that bound E53 were sorted, and within one screening step, sufficient numbers of E53-binding cells were isolated to perform reverse transcription PCR (RT-PCR). The gene encoding the E53-binding protein was identified as a differentially glycosylated isoform of the P-selectin ligand (Fig. 4A). It is worthwhile mentioning that P-selectin ligand is a heavily glycosylated protein, with >50% of the molecular mass made up by carbohydrates. A detailed description of the E53 monoclonal antibody and its ligand will be given elsewhere (C. Ruedl, unpublished data). We further validated the method by isolating CD14-, CD16-, CD19-, and CD32-expressing cells and the respective genes with similar efficiency (data not shown).

Table 1. Quality of the cDNA and viral libraries (mouse brain)

	cDNA library	Viral library
Number of primary clones	1.5×10^6 CFU[b]	10^8 PFU[b]
Average insert size[a]	1.5 kb	1.25 kb
Percentage of full length[a]	40%	30%

[a]Average insert size and percentage of full length was determined from 30 bacterial colonies (cDNA libraries) or by RT-PCR from 30 plaque-purified SIN replicons.
[b]CFU, colony-forming units; PFU, plaque-forming units

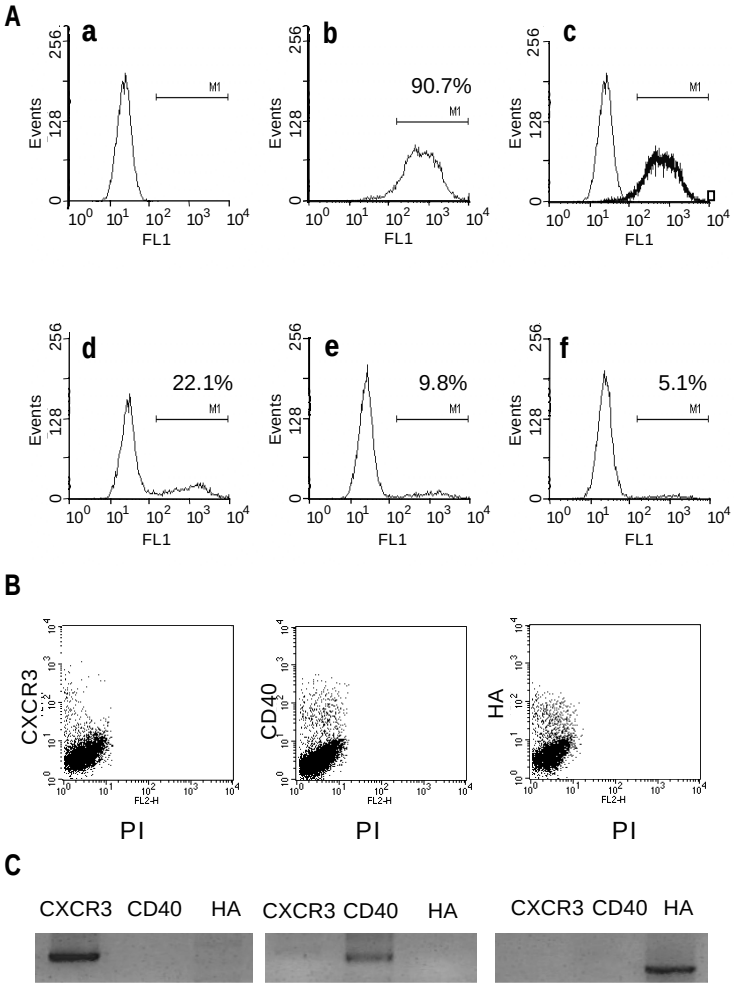

Figure 2. Highly efficient and controlled infection with single SIN replicon particles. (A) Sindbis GFP virus–infected BHK cells were monitored for GFP expression 6 h post-infection by FACS analysis. BHK cells were infected with control virus (negative control) shown in histogram (a) or with SIN replicon particles expressing GFP at MOIs of 1 (b), 0.2 (d), 0.1 (e), and 0.05 (f). In histogram (c), histograms (a) and (b) are overlaid. The single-hit kinetics of Sindbis virus infection is demonstrated by the linear correlation of MOI versus percentage of GFP-positive cells in region M1. All histograms represent 10,000 measured cells. (B) Ten thousand BHK cells were infected with SIN replicon particles expressing CXCR3, CD40, and HA (MOI of 0.03–0.08 each). Samples were stained for expression of the various molecules, and single-cell sorting was carried out by excluding propidium iodide–positive dead cells. (C) Released virus was amplified in 24-well plates; cDNA was generated from infected cells and assessed for the presence of CXCR3, CD40, and HA DNA by PCR. Infected cells that stained positive for one of the proteins exclusively released virus encoding the respective gene. Ten cells were analyzed for each group. One representative experiment of three is shown.

Many sequencing projects have identified a large number of ligands with unknown binding partners. To assess whether DELphi is suited to identify receptors for such ligands, we generated a bait protein composed of a fusion between CD40 ligand (CD40L) and the constant part of human IgG1 (CD40L-Fc). BHK cells were infected at an MOI of 0.2 with a SIN replicon library derived from human spleen and stained with the CD40L-Fc fusion molecules. Single positive cells were sorted and viral vector-encoded genes determined. Out of 20 sorted single cells producing infectious virus, 3 reproducibly bound CD40L and encoded CD40 (Fig. 4B and not shown).

Use of Sindbis virus for a eukaryotic plaque-lift assay. In addition to describing methodology for the identification of cell surface molecules, we also developed a method for isolation of secreted or intracellular molecules. To allow the identification of such molecules, a plaque-lift assay was developed. BHK cells were seeded into petri dishes and infected with 5,000–20,000 control SIN replicon particles expressing no foreign proteins, spiked with recombinant SIN replicon particles expressing either CD40 (membrane), GFP (intracellular), or TNF (secreted) at a ratio of 1:100. Cells were first overlaid with 0.8% agarose and then, 24 h later, a polyvinylidene fluoride (PVDF) membrane. After one day (secreted proteins) or three days (intracellular and membrane proteins), PVDF membranes were assessed for the presence of the respective proteins using monoclonal antibodies (GFP, TNF) or a CD40L-Fc fusion molecule (CD40) (Fig. 5A–C). The number of positive spots on the membrane approximately corresponded to the number of recombinant SIN replicon particles mixed with the control particles (data not shown). In addition, plaques corresponding to spots on the membrane could be picked and, upon initiation of a new round of infection, viruses could specifically be amplified (data not shown). The observation that intracellular proteins can be captured on the membrane indicates that this subset of cellular proteins may also be accessible for protein–protein and possibly even protein–DNA interactions (by using labeled proteins or DNA for probing the membrane). Moreover, the results indicate that it may be possible to use radiolabeled ligands for identification of interaction partners, offering the opportunity to use small molecules for screening.

We next assessed whether the method could be used to isolate genes from libraries. BHK cells infected with SIN replicon particles encoding a mouse spleen cell library were probed for the presence of proteins recognized by the monoclonal antibody E53 (Fig. 5D). Positive plaques were identified and specific virus amplified (Fig. 5E). No positive plaques could be found on cells infected with control virus (Fig. 5F). DNA sequencing confirmed that only clones harboring the P-selectin ligand were selected. Moreover, the frequency of positive plaques roughly corresponded to the frequency of E53-encoding viruses observed by FACS analysis (data not shown).

Comparison with other methods. Our data demonstrate that the method described here permits screening of cDNA libraries with high speed and specificity. This is the result of several features that render our alphavirus-based expression system advantageous for expression cloning: (1) High levels of gene expression are easily obtained at low multiplicities of infection, leading to the expression of one gene per cell. (2) The cDNA library is cloned using standard procedures and does not involve homologous recombination as is the case for libraries constructed with DNA-viruses, such as vaccinia-, baculo-, or adenovirus[8,9,21]. (3) Infectious viruses are readily isolated from infected cells and sufficient virus is generated within 48 h from a single cell to allow sequencing of the library-derived cDNA. Thus, virally infected cells encoding a gene of interest need not be grown from single cells, which is time consuming and difficult, particularly if the encoded gene is toxic. Also, multiple rounds of subcloning are not necessary[22]. (4) SIN replicon titers are somewhat lower for large insert sizes as described for retroviruses[6,23]. This problem can be avoided in our system by generating size-fractionated libraries (data not shown).

A disadvantage of the method in its current form is the fact that SIN virus severely interferes with the host cell metabolism. We are currently addressing this problem by investigating the use of noncytopathic viral variants[24], which may have less effect on the host cells.

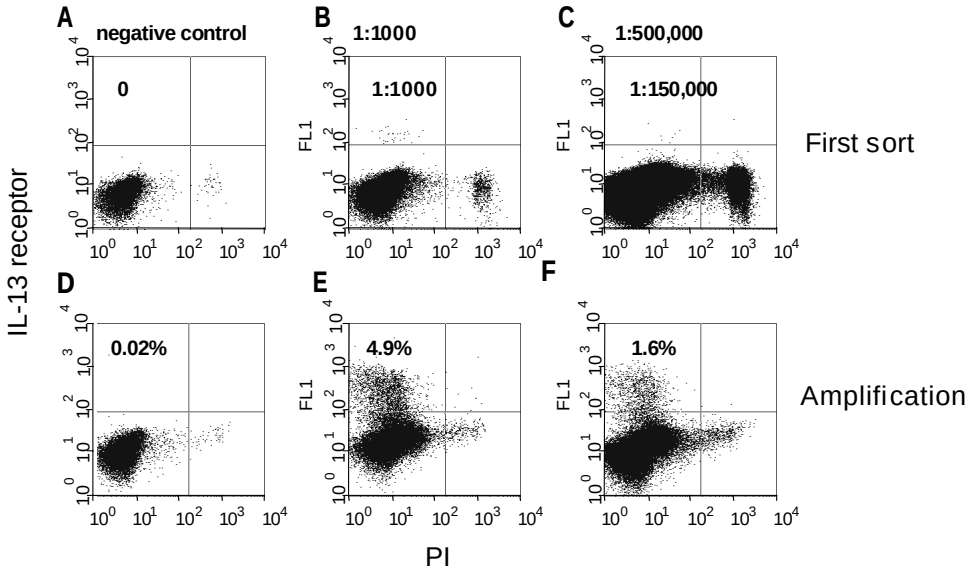

Figure 3. Sorting and recovery efficiency of SIN replicon particle–infected cells. Cells infected with SIN replicon particles expressing the α1-chain of the human IL-13 receptor were diluted in cells infected with control SIN replicon particle at indicated ratios: (A) negative control, (B) 1:1,000 dilution, and (C) 1:500,000 dilution. Upon sorting of IL-13 binding cells into 24-well plates containing a monolayer of BHK cells (A–C), virus spread was observed and IL-13 receptor expression was monitored by flow cytometry 48 h later (D–F). Infected cells were isolated and the IL-13 receptor was identified by RT-PCR (data not shown). In (A) 25,000 cells were analyzed; in (B) 50,000 cells; in (C) 2 x 10^6 cells. Ratios and percentages within the plots refer to observed frequencies of IL-13 receptor positive cells. One representative experiment of three is shown. PI, Propidium iodine.

Our system, like many others, is limited to screening for proteins that function as homomeric polypeptide chains[25]. However, many cytokine receptors, such as IL-6 or IL-13, consist of heteromeric transmembrane proteins. Nevertheless, in many cases, expression of one subunit alone is sufficient for low-affinity binding to ligand[26]. Hence, our single-clone expression system may still be useful for ligand binding (see also Fig. 3). Other difficulties, such as the transient nature of the expression system and recombination between the pSinRepLIB RNA and the helper RNA (ref. 27), are easily solved by the positive selection that occurs in the single cell-sorting step or by plaque purification.

In conclusion, DELphi technology is an alphavirus-based expression screening technology that takes advantage of the SIN virus biology. High levels of expression, single-clone representation of cDNA, and self-amplification of the infectious vector all offer a rapid, high-throughput system. Hence, it is possible to clone ligands and cell surface molecules in a single step from a complex library. The sequencing of the human genome has produced a great number of genes encoding receptors/ligands with unknown binding partners. DELphi may allow for rapid identification of these molecules.

Experimental protocol

Cells and virus constructs. All experiments were carried out with BHK-21 cells cultured as monolayers with HP-1 medium (Cell Culture Technologies, Glattbrugg, Switzerland) supplemented with 5% FCS. The SIN-based expression vector pSinRepLIB (pSINRep5; ref. 19) backbone with a changed multiple cloning site (old, *Xba*I-*Bsp*120I; new, *Xba*I-LPP1-*Pme*I-*Mlu*I-*Mlu*I-*Bsp*120I-LPP2; for LPP1 and LPP2, see below) and the SIN vector pDH-EB encoding the viral structural protein genes (both obtained from S. Schlesinger, University of Washington, St. Louis, MO) were used as a transient expression system. The SIN plasmid DNAs were linearized and RNase-purified by applying a PCR purification kit (QIAquick PCR purification, Qiagen, GmbH, Heidel, Germany). Upon *in vitro* transcription of the viral RNAs (SP6 *in vitro* transcription kit, Invitrogen Inc., Groningen, The Netherlands), 2 μg of each RNA were co-electroporated into 10^7 BHK-21 cells. High-titer SIN replicon stocks (5 x 10^6–3 x 10^7 plaque-forming units (PFU) per ml) were harvested 20 h post-electroporation, and the SIN pools were titrated on BHK-21 cells as described in the plaque-lift assay.

cDNA libraries. Unidirectional cDNA libraries (5′ blunt and 3′ *Not*I) were prepared at LTI (LTI and Gibco BRL, Grand Island, NY). These were then subcloned into a transition vector (pFBLIB2) before the cDNA inserts were excised with with *Stu*I (5') and *Not*I (3') and then cloned into pSinRepLIB (5′ *Pme*I and 3′ *Bsp*120I). Upon ligation, pSRLIB-cDNA libraries were transformed into electrocompetent *Escherichia coli* XL1blue cells and plasmid DNA was isolated from ampicillin-resistant bacterial colonies.

Sorting strategy. Subconfluent (80%) BHK-21 cell layers were infected with recombinant SIN replicon particle pools at an MOI of 0.2 for 2 h. Cells were detached 8 h postinfection with cell dissociation buffer (PBS-EDTA; Gibco BRL). SIN replicon particle–infected cells were incubated for

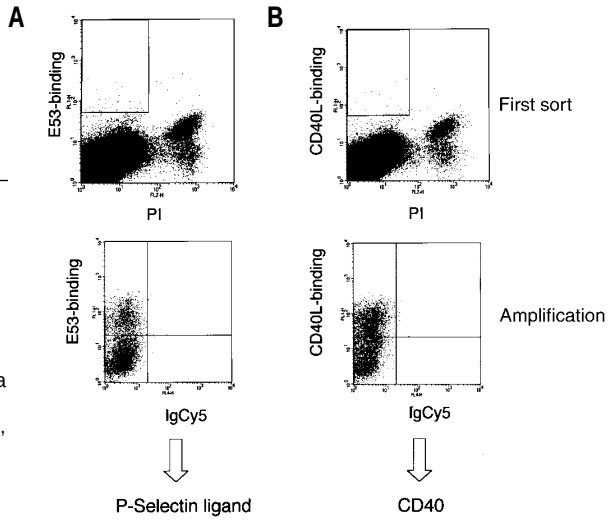

Figure 4. Single-step isolation of ligands for monoclonal antibodies or Fc-fusion molecules. A mouse spleen cDNA library was cloned into pSinRepLIB vector and transformed into high-titer virus pools. Mammalian cells were infected with 0.2 plaque-forming units per cell with control SIN replicon particles or virus encoding the cDNA libraries. (A) Library-infected BHK cells were stained with monoclonal antibody E53, and 171 positive cells were isolated from 2.4 x 10^6 cells. Single cells were sorted into 24 wells containing a subconfluent layer of BHK. (B) Library-infected cells were stained using a CD40L-Fc fusion molecule, and 110 positive cells were isolated from 9 x 10^6 cells. Single cells were sorted into wells of a 24-well plate containing a subconfluent layer of BHK. Upon viral amplification, infected cells of 24 well plates were analyzed for E53 and CD40 surface expression, resulting in positive clones (lower panel). cDNA was generated from positive cells and the sequences of the genes encoded were determined. One representative experiment of three (A) or five (B) is shown. PI, Propidium iodine; IgCy5, Cy5-labeled IgG for exclusion of Fc receptors.

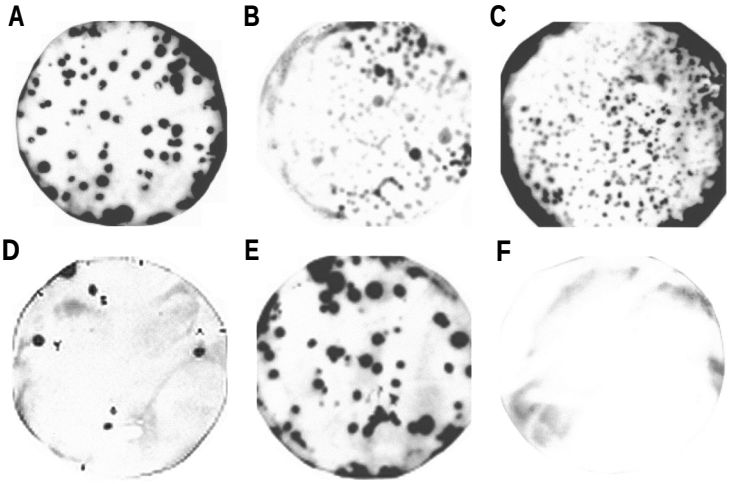

Figure 5. Isolation of genes encoding secreted, intracellular, and membrane proteins. (A) Ten thousand or (B, C) twenty thousand SIN replicon particles, spiked at a ratio of 1:100 with SIN replicon particles expressing TNF (A), GFP (B), and CD40 (C), were seeded into petri dishes containing BHK cells and overlaid with agarose to limit diffusion of virus and proteins. After 24 h, a PVDF membrane was placed on top of the agarose and presence of protein was assessed on the membrane after 24 h (A) or 72 h (B, C) using monoclonal anti-TNF antibody (A), a monoclonal anti-GFP antibody (B), or a CD40L-Fc fusion molecule (C). Recombinant SIN replicon particles particles encoding a mouse spleen library were used in a similar experiment, and membranes were assessed for the presence of proteins recognized by monoclonal antibody E53 (D). Positive plaques were picked, and specific virus was amplified by this procedure as determined by a second plaque-lift assay (E). In (F), a negative control with control virus is shown.

5 min with Fc-Receptor block (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). Indirect immunostaining was performed subsequently according to the ligands used. Fluorescein isothiocyanate (FITC)–conjugated second-stage antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Anti-CXCR3 and anti-TNF antibodies were purchased from PharMingen (San Diego, CA). The CD40 ligand–Fc fusion molecule was produced and purified as described[28]. The immunostained cell pools were then filtered, and dead cells were stained with propidium iodide (PI; 2 μg/ml). FACS was performed on a FACS Vantage flow cytometer (Becton Dickinson, Franklin Lakes, NY) sorting for FITC-positive and PI-negative single cells. These cells were then incubated in a 96-well plate with a fresh layer of 20% confluent BHK-20 cells. Upon virus spread (one to two days post-sorting), the infected cells were tested for the surface expression of the binding partner by FACS analysis.

Plaque-lift assay. BHK-21 cells were seeded into 15 cm petri dishes to generate 80% confluent cell layers. These cells were infected with up to 20,000 PFU for 2 h, before the medium was replaced with a 3 mm layer of HP1 medium containing 0.8% agarose. After 20 h, a PVDF membrane (Immobilon-P; Millipore, Bedford, MA) was applied on top of the agarose layer for 24 h (secreted proteins) and up to 72 h for cytoplasmic and transmembrane protein detection. The membranes were then removed and subjected to standard staining procedures using the monoclonal anti-GFP (Invitrogen, Carlsberg, CA), anti-TNF or E53 antibody or CD40L-fusion molecules, and developed using the ECL protocol (Amersham Pharmacia Biotech, Buckinghamshire, UK).

RT-PCR and sequencing. Total RNA was isolated from BHK-21 cells (5×10^4 up to 10^6) that were infected with SIN replicon and found positive for binding of a defined ligand or antibody (RNeasy protocol; Qiagen GmbH). The cDNA inserts were amplified by an RT-PCR (Thermoscript RT-PCR; Gibco BRL) using isolated total RNA (from 25,000 up to 150,000 cells) and the LPP2 primer (see below) for the reverse transcription (50°C for 1 h). The LPP1 (5′-ATACGACTCA CTATAGGGAGACAGG-3′) and LPP2 (5′ACAAATTGGACTAATCGATGGC-3′) primers were then used for the PCR cycles (1 min 30 s at 94°C, 30× 94°C 30 s, 60°C 30 s and 72°C for 3 min 30 s, before an additional 5 min at 72°C). The RT-PCR products were cloned into pGEM-T vector (according to Clontech, Palo Alto, CA) and sequenced using the SP6 and T7 primers.

Acknowledgments

We thank Dr Gary Jennings and Mark Dyer for carefully reading the manuscript and Prof. Sondra Schlesinger for many helpful discussions and for providing the plasmids pSinRep5 and pDH-EB. This article is dedicated to the memory of Prof. James E. Bailey, who passed away during the preparation of the manuscript.

Received 13 December 2000; accepted 21 June 2001

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