15
Use and Applications of Subtractive Antibody Screening

PERRY E. BICKEL[1], HARVEY F. LODISH[2] AND PHILIPP E. SCHERER[3*]

[1]*Departments of Medicine and Cell Biology and Physiology, Washington University School of Medicine, Campus Box 8127, St. Louis, MO 63110, U.S.A.,* [2]*The Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology, Cambridge, MA 02142, U.S.A. and* [3]*Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY 10461, U.S.A.*

Introduction

Information about where and when genes are expressed is critical to understanding the function of the proteins that they encode in both health and disease. This concept is driving robust technology development in the field of functional genomics. Gene chips and other gene array systems permit the expression of thousands of genes to be surveyed simultaneously. A limitation of these approaches is that differential expression is detected at the level of RNA. Due to regulatory mechanisms that operate at the translational and post-translational levels, the absolute amounts of mRNA expression of a particular gene may not reflect the levels of its protein. Nevertheless, it is the level of protein that is ultimately more informative biologically in most cases. Another limitation is that gene expression data do not reveal important functional details, such as secondary modifications of proteins or their subcellular localization. The emerging field of proteomics addresses these limitations by working at the protein level to identify differential expression. By this approach, proteins in complex mixtures, such as cell lysates, are separated from one another chromatographically or electrophoretically, and then identified by such methods as microsequencing or mass spectroscopy, both of which are costly and labour intensive.

We developed subtractive antibody screening (SAS) (Scherer *et al.*, 1998) as a tool for functional proteomics (*Figure 15.1*). Our goal was to create a method by which particular subsets of differentially expressed proteins could be identified and their corresponding cDNAs cloned systematically. Further, we required that the method not depend on high cost equipment or services. In short, SAS relies on the generation

*To whom correspondence may be addressed (scherer@aecom.yu.edu)

Abbreviations: SAS, subtractive antibody screening; SDS-PAGE, sodium dodecyl sulphate polysacrylamide gel electrophoresis.

Biotechnology and Genetic Engineering Reviews – Vol. 17, August 2000
0264–8725/00/17/417–430 $20.00 + $0.00 © Intercept Ltd, P.O. Box 716, Andover, Hampshire SP10 1YG, U.K.

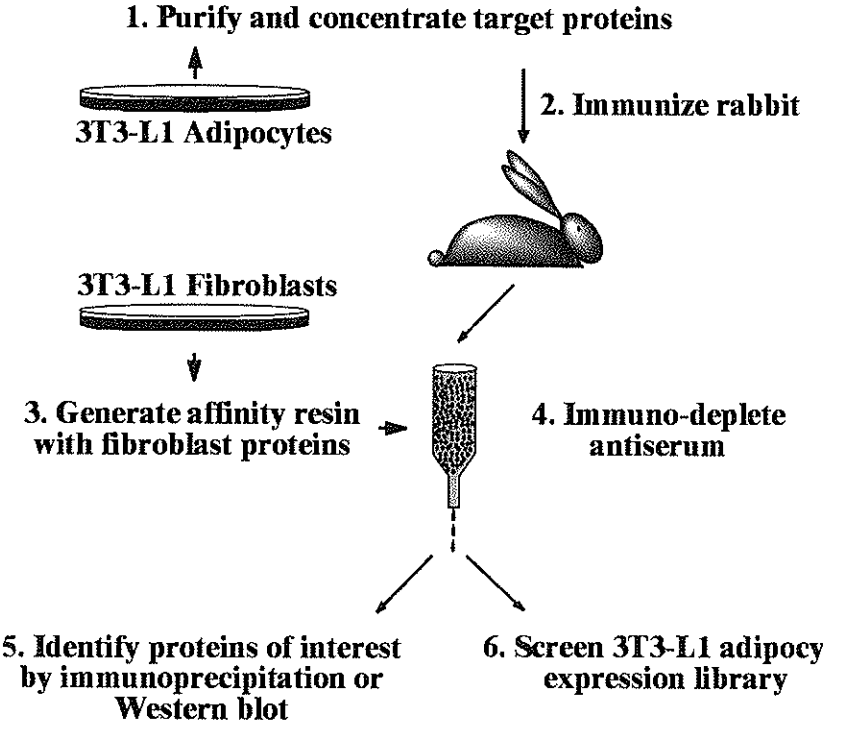

Figure 15.1. Outline of the SAS approach using 3T3-L1 adipocyte secretory or cell surface proteins as target antigens.

of polyclonal antisera obtained by injection of a complex mixture of antigens. Similar approaches have been described that utilize polyclonal antisera against complex antigens to screen phage expression libraries (Mierendorf *et al.*, 1987). We have extended this technology by introducing a subtractive process that increases the specificity of the antiserum for target proteins. In SAS, the complex antiserum is selectively depleted of undesirable antibodies by employing multiple depletion matrices to generate an antiserum that is highly enriched in antibodies that recognize differentially expressed proteins. These antisera can be used for functional studies, as well as for expression cloning approaches. Because our research interest is centred around the identification of auto-, para- and endocrine factors released by adipocytes, we initially chose to focus on adipocyte-specific proteins that are either secreted (ie hormones, cytokines, growth factors, enzymes, and matrix proteins) or associated with the plasma membrane (ie receptors, transporters, channels, signalling molecules, and adhesion molecules). These subsets of proteins are attractive targets for drug development due to their accessibility in the extracellular space. However, as will be apparent, any subset of proteins that can be isolated at high purity could serve as the target antigens for SAS.

This review will describe subtractive antibody screening with an emphasis on the parameters critical to its successful application. We will discuss potential applications of SAS and modifications of the protocol that would facilitate those applications.

Finally, SAS will be compared and contrasted to other subtractive antibody approaches that have been described in the literature.

Outline of the SAS technology

The first, and most critical, step of SAS is the purification of target proteins. These proteins are combined with RIBI adjuvant (RIBI ImmunoChem Research, Hamilton, MT, U.S.A.) and used to immunize rabbits, or another animal suitable for the production of polyclonal antisera. In our experience, rabbits immunized with a complex mixture of proteins generate a robust titre of polyclonal antibodies within two months. The quality of the resulting antisera is then evaluated by western blotting or immunoprecipitation of the target proteins or by immunocytochemistry/immunohistochemistry performed on relevant cells/tissues. If satisfactory recognition of the target proteins compared with preimmune serum is not achieved, alternative strategies for antigen preparation have to be considered. Such strategies include increasing the amount of antigen used per injection and/or presenting the antigens in a denatured conformation by pre-treatment with a denaturing reagent such as sodium dodecyl sulphate. In order to produce an antiserum that is relatively enriched in antibodies that recognize the target subset of differentially expressed proteins, the serum is progressively depleted of undesired antibodies by passing it over solid supports to which irrelevant antigens have been fixed. After depletion, the antiserum is evaluated as above, with an emphasis on specificity for target proteins versus non-target proteins. Multiple rounds of subtraction using various forms of affinity matrices are used as necessary to generate an antiserum that specifically recognizes the desired targets.

The subtractive antiserum can be used directly in a cloning project to immunoscreen bacteriophage lamda cDNA expression libraries. Upon completion of the various depletion steps, the 'signal-to-noise' ratio is usually quite good, yielding less than 20% false positives (ie known non-target proteins) in immunoscreening procedures (Scherer *et al.*, 1998).

With this approach, we cloned a number of known adipocyte secretory and cell surface proteins, as well as novel clones that we are currently further characterizing (*Table 15.1*). Additionally, the subtractive antiserum is a valuable tool for the identification of proteins that demonstrate desired characteristics. For example, using a subtractive antiserum raised against adipocyte secretory proteins, we were able to

Table 15.1. Clones isolated from SAS screens

Screen for secreted proteins	Screen for cell surface proteins
Acute phase response proteins	Enzymes
Complement factor C3	Lipoprotein lipase
α1 acid glycoprotein	Copper amine oxidase
Serum amyloid A (SAA)	
Extracellular matrix proteins	Receptor-like protein
Fibronectin	HDL-binding protein/vigilin
Osteonectin/SPARC	
Type VI (α3) collagen	
5 novel clones	Cell-cell interaction protein
	Basigin/HT7/EMMPRIN
	5 novel clones

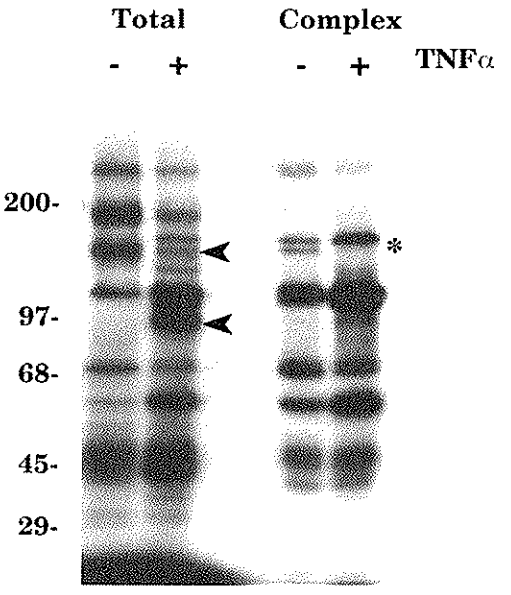

Figure 15.2. Use of the complex antiserum raised against adipocyte secretory proteins for the immunoprecipitation of ^{35}S-labelled secretory proteins harvested before ('-') or after ('+') treatment for 12 hrs with TNFα. The complex antiserum used for this experiment had not been subjected to subtractive procedures. For comparison, the total protein content of the supernatant before the immunoprecipitation is also shown. Note that the complex antiserum recognizes most of the proteins found in total supernatant, but selectively depletes (arrowheads) or enriches (asterisks) for specific proteins. Upon analysis with two-dimensional gel electrophoresis (isoelectric focusing followed by SDS-PAGE), the increased resolution allows the identification of many more individual protein spots whose expression is differentially regulated (not shown).

describe a number of proteins whose secretion was regulated by insulin (Scherer *et al.*, 1998) or TNFα (*Figure 15.2*). Once detected as bands on a gel or blot, these proteins can be identified further by traditional protein purification techniques, or by first isolating antibodies to these proteins from the subtractive antiserum as described below.

Critical parameters

PREPARATION OF IMMUNOGEN

A major advantage of SAS is that any complex mixture of proteins that can be isolated with relative purity may be used as the immunogen. This gives the technique wide applicability to address many questions of differential protein expression. The purity of the immunogen will influence how much background is seen when the resulting antiserum is used in the initial rounds of immunoscreening. To a large degree, antibodies in the complex antiserum that are generated against non-target proteins can be subtracted out by incubation of the antiserum with solid supports, either beads or membranes, to which the background antigens have been coupled. However, a given background antigen that is particularly immunogenic will create 'noise' in the immunoscreen that is difficult to overcome and may reduce the relative titre of antibodies against less immunogenic target proteins.

Our experience with immunoscreening for adipocyte secreted proteins illustrates these points. We collected media conditioned by 3T3-L1 adipocytes, concentrated the proteins, and mixed the concentrated media with RIBI adjuvant. In order to eliminate the generation of antibodies against components of foetal calf serum, the adipocytes were washed extensively and incubated in serum-free medium, both with and without supplemental insulin prior to the collection of secretory proteins. These conditions presumably led to some cell death and release of intracellular contents into the medium because our initial screens with the resulting antiserum contained a high titre of antibodies against intracellular proteins of high immunogenicity (eg heat shock proteins). In contrast, when we used purified plasma membrane proteins as the immunogen, the antibody titre against such highly immunogenic intracellular proteins was minimal. In this context, the plasma membrane protein immunogen had two advantages over the secreted protein immunogen. First, the adipocytes spent a minimal amount of time in serum-free conditions. Second, the method of isolation – biotinylation of the target proteins (further discussed below) – permitted their purification on a solid support with extensive washes. In summary, the target proteins for SAS must be amenable to purification from the bulk of cellular proteins by a method that minimizes contamination from non-target fractions.

Does the conformational state of the immunogen influence the titre of the antiserum produced? We have used purification and concentration techniques that yield proteins in both their native and partially or fully denatured state. The use of concentrator units that rely on centrifugation preserves the native protein conformation during the concentration process. Precipitation of the collected proteins in 20% (w/v) trichloroacetic acid/80% acetone and resolubilization in 1% sodium dodecyl sulphate in Tris buffer results in both concentration and denaturation of the proteins. The presence of denatured proteins in the immunogen has two advantages. First, if the resulting antiserum is used for immunoscreening, the antibodies are likely to recognize the partially denatured state of the fusion proteins produced in bacteria. Second, denatured proteins are more likely to be immunogenic even if their primary amino acid sequence is highly conserved between the target species and the immunized host species. However, if the antiserum is to be used for immunoprecipitations under non-denaturing conditions, for screening of cDNA expression libraries in mammalian cells or for neutralization studies, it is essential to have antibodies available that recognize native conformations of proteins.

IMMUNIZATION

SAS relies on the generation of an immune response in a host animal. The degree of response depends upon many factors, including the antigenicity of the immunogen, the amount of immunogen, the immunization protocol, and the phylogenetic relationship of target and host species. We have used from 250 to 750 micrograms of immunogen per boost, with good results. For some protein subsets, it may be impossible to obtain milligram quantities for immunizations. In such cases, alternative strategies that rely on subtraction at the level of cDNA may be more appropriate for the identification of differentially expressed mRNAs. The choice of the proper host for the generation of the antibody is an important parameter as well. While we have relied predominantly on rabbits, the use of phylogenetically more distant hosts

may be an important factor in the generation of an effective high-titre preparation. Particularly in the context of raising antibodies against secretory and cell surface proteins of mammalian cells, chickens are suitable hosts due to their phylogenetic distance, the ease with which antibodies can be generated, and the very high yield of IgY's produced. We have successfully used chickens as host species for the generation of a complex antiserum against mouse adipocyte plasma membrane preparations.

ENRICHMENT OF ANTIBODIES AGAINST TARGET PROTEINS

We found the immunodepletion of antibodies against non-target proteins from the complex antiserum to be critical for its subsequent use in cloning. Immunodepletion should be done extensively and in as many different ways as practically possible. Because our target proteins were adipocyte secretory and cell surface proteins, we depleted the complex antisera on solid supports, specifically CNBr-Sepharose (Pharmacia & UpJohn Diagnostics, Kalamazoo, MI, U.S.A.) to which we had coupled preadipocyte proteins. The antiserum against adipocyte cell surface proteins was also incubated with live, intact preadipocytes in order to deplete antibodies that would recognize the exofacial domains of non-target plasmalemmal proteins in their native state. Another particularly effective method of depletion is the incubation of the antiserum with membranes coated with phage plaques expressing recombinant antigen to non-target proteins. Such undesired activities can easily be identified in an initial pilot screen as described below. Clonal isolates of phage expressing undesired fusion proteins can be used to prepare filters for depletion. This represents an unlimited source of fusion protein, and quantitative depletion of undesired antibodies can be achieved.

PREPARATION OF BACTERIOPHAGE CDNA LIBRARY AND IMMUNOSCREENING

No matter how high the quality of the subtractive antiserum, poor cloning results will be obtained if insufficient care is paid to the cDNA expression library. We generated an oligo-d(T)-primed library from 3T3-L1 adipocyte polyadenylated RNA in the vector λEXLOX (Novagen Inc., Madison, WI, U.S.A.). This vector system has key features that permit directional cloning of inserts and reliable generation of plasmid subclones using the Cre-loxP site-specific recombination system. A more recent version of this vector (λSCREEN-1) introduces a histidine tag into the fusion protein which facilitates the rapid isolation of the fusion protein for use in the immunopurification of monospecific antibodies from the complex polyclonal antiserum, as described below. Provision is also made for directional cloning of random hexamer-primed libraries, which may have an advantage over oligo d(T)-primed libraries for the purpose of immunoscreening a library of protein epitopes. Because small cDNA inserts (300–1,000 base pairs) are adequate in a library to be used for immunoscreening, cDNA libraries generated by various subtractive protocols may be particularly well suited for screening in order to increase cloning specificity for targets.

After the initial round of screening, it becomes clear whether or not there are particular nuisance clones that are recognized by antibodies in the subtractive antiserum. If so, then phage particles for these clones can be pooled, amplified in

bacteria, and their respective fusion proteins transferred to a membrane by plaque lift. Incubation of the subtractive antiserum with multiple such membranes systematically depletes it of these unwanted activities, and also of antibodies that crossreact with the bacterial proteins that are also present on the membranes.

Quantitative aspects

Whether or not an immunological response against a specific protein component in a mixed population of polypeptides will be elicited depends on both the relative abundance of the molecule, as well as its immunogenicity. The very strength of SAS relies on the fact that less abundant, but more immunogenic, proteins can trigger an immune response sufficiently high to allow the cloning of the respective cDNA from phage expression libraries. As can be seen from *Figure 15.2*, the majority of the proteins trigger antibody production as judged from the mostly overlapping patterns obtained upon comparison of the total complement of secretory proteins versus the pattern obtained by immunoprecipitation with the complex antiserum.

While it is impossible to say how many copies of a given protein have to be present within a cell to trigger a response, the spectrum of clones isolated to date suggests that moderately expressed clones, such as cell surface receptors and signalling molecules, can be isolated by this method as well (see *Table 15.1* and our unpublished observations).

Once an antiserum is generated, it is subjected to immunodepletion with the respective protein pool isolated from a non-target source, ie an undifferentiated cell, a non-transformed cell type, a different subcellular fraction, etc. This immunodepletion is generally achieved by using an excess of these irrelevant antigens. However, caution must be exercised with respect to how much irrelevant antigen is used for the depletion. Because irrelevant antigen mixtures may be contaminated with low levels of target antigen, antibodies that recognize a desired protein expressed even at low levels in the precursor cell may be removed during the subtraction procedure, which would preclude the cloning of its cDNA by SAS.

Target populations of immunogens

As indicated above, any population of polypeptides with a common subcellular or extracellular localization or biochemical property can serve as a complex antigen mixture for the production of antibodies. The higher the purity of the target population, the better the quality of the resulting antibody preparation. The following paragraphs describe some of the possible targets that we have successfully used for a number of different applications.

SECRETORY PROTEINS FROM TISSUE CULTURE CELLS

As described in the initial description of the technology, we have used serum-free tissue culture supernatants from 3T3-L1 adipocytes for the generation of our initial antiserum. It is important that the cell-type of choice can be maintained for several hours in the absence of serum, since the collection of secretory proteins in the cell supernatant should occur in an otherwise protein-free environment. During or before

collection of supernatant, cells can be exposed to effectors of choice, such as drugs, growth factors, or hormones.

PLASMA MEMBRANE PROTEINS

Plasma membranes prepared from isolated adipocytes have been used to immunize host animals for the production of polyclonal antisera, and these antisera have been used for immunoprecipitation, immunoblotting, and cytotoxicity experiments (Flint, 1998; Tume, 1991). A number of different protocols are available for the specific isolation of plasma membrane proteins. Conventional purification protocols rely on the generation of plasma membrane vesicles that are purified using equilibrium and velocity sedimentation gradient centrifugation (Jenkins *et al.*, 1994; Magocsi and Penniston, 1991; Tsutsumi *et al.*, 1994). An alternative, more powerful approach relies on the cell surface derivatization of intact cells with an N-hydroxy-succinimid-derivatized biotin. If performed at 4°C to prevent endocytosis, this is a very effective means to isolate a relatively pure population of molecules localized to the plasma membrane. Following derivatization and quenching of excess activated biotin, the cells can be lysed, and biotinylated proteins can be isolated using a streptavidin column. Given the very strong affinity of the biotin-streptavidin interaction, a wide variety of different stringencies can be used for the washing conditions. The washes can be performed under native conditions that preserve some of the interactions with cytoplasmic signalling molecules. Alternatively, washes can be performed under very stringent conditions because the streptavidin-biotin interaction is stable in 0.5% SDS, which allows removal of the bulk of associated molecules that are not directly biotinylated (Scherer *et al.*, 1998).

LECTIN-BASED AFFINITY PURIFICATION OF GLYCOSYLATED PROTEINS

An alternative method for enrichment of glycosylated molecules which represent the subset of proteins exposing domains facing the exoplasmic side of the plasma membrane as well as the luminal side of the secretory pathway entails the use of a variety of different matrices containing immobilized lectins. To cover the widest possible spectrum of sugar modifications, broad-spectrum lectins can be used, such as Wheat Germ Agglutinin (specific for terminal *N*-acetylglucosamine) or *Lens culinaris* Agglutinin (specific for α-Mannopyranosyl residues). Elution is best performed with the corresponding free sugar residues. A large number of different lectin resins are commercially available. Alternatively, sugar residues on the cell surface can be activated in intact cells with periodate, which causes the oxidation of vicinal hydroxyls to form aldehydes. A number of suppliers offer hydrazide-derivatized biotin (eg Pierce Inc., Rockford, IL, U.S.A.) that can be used to form covalent hydrazone bonds with the activated sugar moieties. If need be, conditions can be chosen such that specific sugar residues are preferentially targeted. Sialic acid residues can be oxidized selectively (O'Shannessy *et al.*, 1987). Alternatively, neuraminidase treatment of intact cells will yield terminal galactose residues, which can be selectively oxidized with galactose oxidase to create aldehydes on these sugars that will specifically react with the biotin-hydrazide (Bayer *et al.*, 1988; Roffman *et al.*, 1986; Skutelsky and

Bayer, 1983). These specifically biotinylated proteins can then be isolated conventionally with immobilized streptavidin, as described above.

CELL TYPE AND DIFFERENTIATION SPECIFIC ANTIGENS

Independent of the method of antigen isolation, many different combinations are available for the choice of the target cell versus depletion matrix. Possible applications for the use of SAS include the generation of tumour stage-specific markers for diagnostic purposes. This critically depends on the availability of a relatively pure population of tumour cells at a defined stage. These cells are then dispersed and allowed to recover as a single cell suspension, or as attached cells in tissue culture. They can then be derivatized by one of the techniques described above, purified, and used as antigen. The parent tissue from which the tumour arose can serve as a depletion matrix. Similarly, other cell lines that can be terminally differentiated in tissue culture are suitable for the identification of pre- or post-differentiation markers.

Use of complex antisera in the identification of partner proteins in crosslinking assays

Beyond the obvious application of expression cloning, complex antisera offer a powerful tool for the identification of unknown proteins crosslinked to a known target protein. Crosslinking approaches have proved useful in identifying pairs of molecules that interact specifically, and in characterizing the architecture of multicomponent complexes by establishing near-neighbour relationships. Many crosslinkers are commercially available that differ with respect to their reactivity for specific residues, the length of the spacer region between the two reactive groups, and overall hydrophobicity/hydrophilicity of the crosslinker (Scherer and Krieg, 1991). The appropriate experimental conditions that successfully generate a crosslinked product have to be established in every case. While the generation of a crosslinked product can reveal the relative molecular mass of an interacting protein in a first approximation, chemical crosslinking is an inherently inefficient technique (on average only ~ 1% crosslinking efficiency). It is usually a formidable task to establish the identity of a crosslinked protein using even the most advanced protein sequencing techniques. Complex antisera have successfully been used for the sequence determination of subpicogram quantities of crosslinked products. We have described the generation of a complex antiserum against a purified preparation of mitochondrial inner membranes (Scherer *et al.*, 1992). In an attempt to identify components of the mitochondrial protein translocation machinery, a radiolabelled mitochondrial precursor was translocation arrested and its protein neighbours probed with chemical crosslinkers. Crosslinked products were indeed generated under these conditions. The complex antiserum raised against inner mitochondrial components was used for an immunoprecipitation assay and specifically immunoprecipitated the crosslinked product, which suggested that the antiserum contained an antibody directed against the crosslinked partner protein. Large-scale preparations of solubilized inner mitochondrial membrane proteins were subjected to column chromatography. The presence of the target protein in a specific fraction from the chromatography column was determined by the following indirect assay. An aliquot from each of the various

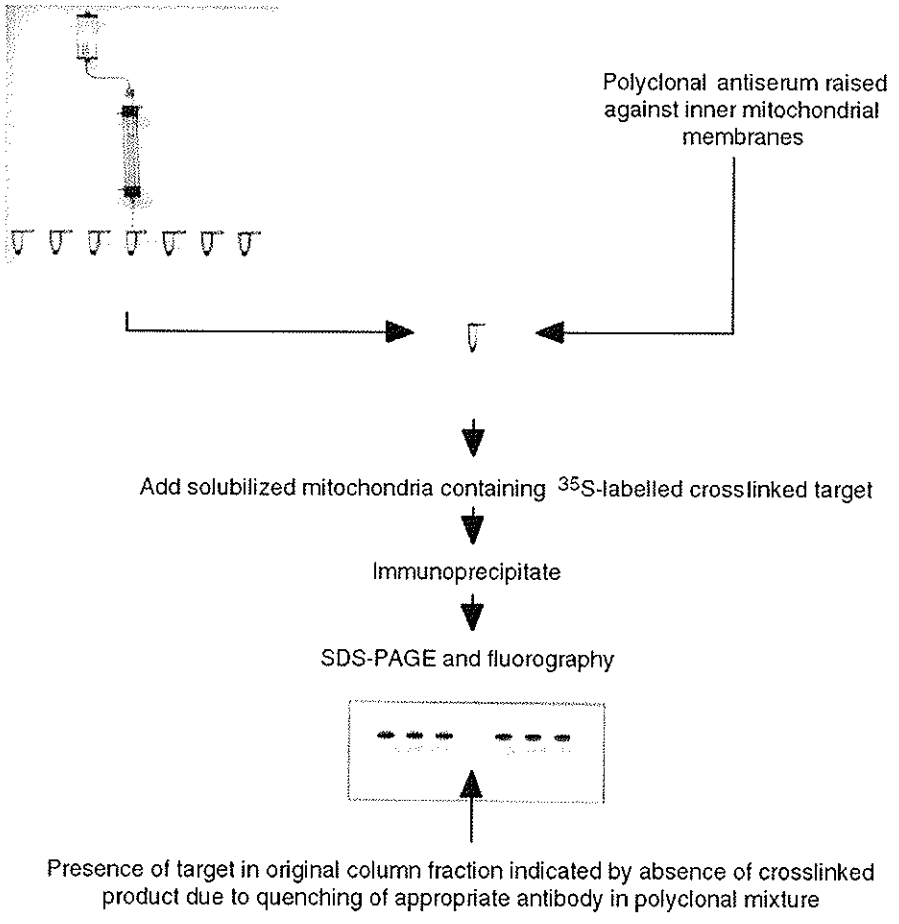

Figure 15.3. Schematic representation of 'quenching assay' employed for the identification and purification of components that are part of crosslinked complexes.

column fractions was mixed with an aliquot of the complex antiserum. If the target protein was present within a given fraction, it 'neutralized' the antibody of interest. The 'quenched' antibody preparations were in turn used to immunoprecipitate a series of labelled extracts containing crosslinked product. Excess unlabelled target protein from the purification neutralized the target antibody, thereby abolishing its ability to immunoprecipitate the crosslinked, labelled band that contained the target protein (see schematic representation in *Figure 15.3*). The *presence* of the target protein in a given fraction was detected by the *absence* of the crosslinked product in the immunoprecipitations. This assay can be used to follow the purification of the target protein through the course of several column fractionations until a homogenous protein fraction is obtained.

Possible purification strategies for the isolation of specific antibodies from the complex antiserum

One of the attractive features of SAS is the immediate availability of an antibody

against a target of interest. As discussed in the first section, if a novel cDNA is isolated by expression cloning with a phage library, the antibody can be directly affinity purified on the corresponding plaques or - upon purification of the corresponding fusion protein – on an affinity column. In analogy to the 'quenching assay' described above, the complex protein mixture used for the generation of the antiserum can be separated by column chromatography. The resulting fractions can be immobilized on a resin (such as CNBr-Sepharose™) and used as affinity matrices for the subfractionation of the complex antiserum. Antibodies can be eluted off those resins and used in the assay of choice. An alternative approach is the use of preparative SDS-polyacrylamide gels run with the protein extract of choice, followed by transfer of the protein to nitrocellulose. The nitrocellulose can then be subdivided into smaller strips according to various molecular weight regions. These strips can be used for affinity purification or depletion to allow a first step towards subfractionation of the antiserum (Scherer *et al.*, 1992).

Other subtractive antibody approaches

Technology that permits the display of fusion proteins on the coat of filamentous phage has led to the construction of large libraries of phage capable of presenting heavy and light chain variable domains. The V gene regions used to produce these libraries either have been derived from harvested lymphocytes of immunized or naïve donors ('natural repertoires') or have been constructed *in vitro* ('synthetic repertoires'). Such libraries of phage monoclonal antibodies (mAb) have been subjected to selection on solid-phase media to yield phage that demonstrate desired binding specificities. This process has been used to generate monospecific antibodies against purified antigens. The method of selection tends to determine the robustness of the selected phage antibodies for downstream applications. The phage antibody technology has been described in reviews (Griffiths and Duncan, 1998; Winter *et al.*, 1994), and we will not discuss its features in depth. However, phage antibodies have been subjected to both positive and negative selection and, therefore, are relevant to our discussion of subtractive antibody techniques. For example, in order to isolate phage mAb specific to thymus stromal cells, Van Ewijk *et al.* (1997) absorbed a phage antibody library with thymocytes and spleen cells before and during positive selection of phage on thymic fragments. The selected phage were eluted from the tissue fragments by incubation in low pH buffer and amplified in bacteria. Three phage mAbs were isolated that, by immunohistochemistry, detected antigens of thymic stromal cells but not of lymphoid cells. In another application, phage mAbs were positively selected from a library by binding to foetal haemoglobin (HbF) after preclearing with adult haemoglobin (HbA) (Parsons *et al.*, 1996). A phage antibody was isolated that could differentiate HbF from HbA by immunocytochemistry and flow cytometry.

A major advantage of subtractive phage antibodies is that, once a phage antibody with sufficient target affinity and specificity is isolated, a stock of it can be generated in perpetuity. Also, the DNA that encodes the selected variable domains can be re-engineered to produce whole immunoglobulin, or can be subjected to saturation mutagenesis and then reselected to produce higher affinity binders (Griffiths and Duncan, 1998). In comparison to polyclonal antibodies generated in immunized

hosts, however, phage antibodies are not as consistently robust for downstream applications such as immunoscreening and immunoprecipitation.

Phage-selected polyclonal antibody libraries (PCALs)

A promising new technology, developed by Sharon and colleagues (Den *et al.*, 1999; Sarantopoulos *et al.*, 1994), enables the perpetuation of standardized mixtures of polyclonal antibodies specific for target antigen(s). In this system, the genetic material encoding Fab fragments is recovered from the B lymphocytes of immunized hosts by reverse transcription and polymerase chain reaction (RT-PCR) using established primer sets. The amplified cDNAs are cloned into a phage display vector and then expressed as fusion proteins on the surface of the phage coat. The resulting phage libraries are subjected to positive selection by binding to target antigens and to negative selection by binding to non-target antigens. Selected phage particles are amplified in bacteria, and the selected antibody genes are transferred *en masse* from the recovered phage vector population to a mammalian vector that provides complete constant region genes and appropriate transcription regulatory elements for expression of whole, glycosylated IgG or IgA antibodies in myeloma cells. Sharon and colleagues have used this technique to generate polyclonal antibody libraries specific for ovarian and breast carcinoma cells and for the protozoan parasite *Cryptosporidium parvum* (Baecher-Allan *et al.*, 1999; Santora *et al.*, 2000; Sharon *et al.*, 2000; and personal communication). The PCAL technology appears to combine the robustness of subtractive polyclonal antibodies (high avidity, low likelihood of antigen 'escape variants', and efficient mediation of effector functions) with the advantages of monoclonal antibodies (unlimited supply of standardized reagents). Whether the PCALs can be used to clone novel cell-specific genes is unknown.

Conclusions

Antibody-based strategies that rely on a mixture of antibodies recognizing a population of antigens with selected properties will become an increasingly relevant tool at a time when several genomes have been, or are about to be, fully sequenced. The focus is shifting towards a functional characterization of these genomes at the protein level. SAS with conventional polyclonal antibodies or with recombinant, phage-based antibodies offers a relatively simple, but extremely powerful, technique for the rapid identification and initial characterization of a wide variety of target proteins.

Acknowledgements

We would like to thank our colleagues for sharing unpublished observations with us.

References

BAECHER-ALLAN, C.M., SANTORA, K., SARANTOPOULOS, S., DEN, W., SOMPURAM, S.R., SHARON, J., CEVALLOS, A.M., BHAT, N. AND WARD, H. (1999). Generation of a polyclonal Fab phage display library to the protozoan parasite Cryptosporidium parvum. *Combinatorial Chemistry & High Throughput Screening* **2**, 319–325.

BAYER, E.A., BEN-HUR, H. AND WILCHEK, M. (1988). Biocytin hydrazide - a selective label for sialic acids, galactose, and other sugars in glycoconjugates using avidin-biotin technology. *Analytical Biochemistry* **170**, 271–281.

DEN, W., SOMPURAM, S.R., SARANTOPOULOS, S. AND SHARON, J. (1999). A bidirectional phage display vector for the selection and mass transfer of polyclonal antibody libraries. *Journal of Immunological Methods* **222**, 45–57.

FLINT, D.J. (1998). Effects of antibodies to adipocytes on body weight, food intake, and adipose tissue cellularity in obese rats. *Biochemical and Biophysical Research Communications* **252**, 263–268.

GRIFFITHS, A.D. AND DUNCAN, A.R. (1998). Strategies for selection of antibodies by phage display. *Current Opinion in Biotechnology* **9**, 102–108.

JENKINS, T.C., THIES, E.J. AND FOTOUHI, N. (1994). Dietary soybean oil changes lipolytic rate and composition of fatty acids in plasma membranes of ovine adipocytes. *Journal of Nutrition* **124**, 566–570.

MAGOCSI, M. AND PENNISTON, J.T. (1991). Oxytocin pretreatment of pregnant rat uterus inhibits Ca2+ uptake in plasma membrane and sarcoplasmic reticulum. *Biochimica et Biophysica Acta* **1063**, 7–14.

MIERENDORF, R.C., PERCY, C. AND YOUNG, R.A. (1987). Gene isolation by screening lambda gt11 libraries with antibodies. *Methods in Enzymology* **152**, 458–469.

O'SHANNESSY, D.J., VOORSTAD, P.J. AND QUARLES, R.H. (1987). Quantitation of glycoproteins on electroblots using the biotin- streptavidin complex. *Analytical Biochemistry* **163**, 204–209.

PARSONS, H.L., EARNSHAW, J.C., WILTON, J., JOHNSON, K.S., SCHUELER, P.A., MAHONEY, W. AND MCCAFFERTY, J. (1996). Directing phage selections towards specific epitopes. *Protein Engineering* **9**, 1043–1049.

ROFFMAN, E., MEROMSKY, L., BEN-HUR, H., BAYER, E.A. AND WILCHEK, M. (1986). Selective labeling of functional groups on membrane proteins or glycoproteins using reactive biotin derivatives and 125I-streptavidin. *Biochemical and Biophysical Research Communications* **136**, 80–85.

SANTORA, K.E., SARANTOPOULOS, S., DEN, W., PETERSEN-MAHRT, S., SOMPURAM, S.R. AND SHARON, J. (2000). Generation of a polyclonal fab phage display library to the human breast carcinoma cell line BT-20.*Combinatorial Chemistry & High Throughput Screening* **3**, 51–57.

SARANTOPOULOS, S., KAO, C.Y., DEN, W. AND SHARON, J. (1994). A method for linking VL and VH region genes that allows bulk transfer between vectors for use in generating polyclonal IgG libraries. *Journal of Immunology* **152**, 5344–5351.

SCHERER, P.E. AND KRIEG, U.C. (1991). Crosslinking reagents as tools for identifying components of the yeast mitochondrial protein import machinery. *Methods in Cell Biology* **34**, 419–426.

SCHERER, P.E., MANNING-KRIEG, U.C., JENO, P., SCHATZ, G. AND HORST, M. (1992). Identification of a 45-kDa protein at the protein import site of the yeast mitochondrial inner membrane. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 11930–11934.

SCHERER, P.E., BICKEL, P.E., KOTLER, M. AND LODISH, H.F. (1998). Cloning of cell-specific secreted and surface proteins by subtractive antibody screening. *Nature Biotechnology* **16**, 581–586.

SHARON, J., SARANTOPOULOS, S., DEN, W., KAO, C., BAECHER-ALLAN, C.M., SANTORA, K.E., SOMPURAM, S.R., PETERSEN-MAHRT, S. AND WILLIAMS, B.R. (2000). Recombinant Polyclonal Antibody Libraries. *Combinatorial Chemistry & High Throughput Screening* **3**, 185–196.

SKUTELSKY, E. AND BAYER, E.A. (1983). Cell-type-related segregation of surface galactosyl-containing components at an early developmental stage in hemopoietic bone marrow cells in the rabbit. *Journal of Cell Biology* **96**, 184–190.

TSUTSUMI, T., KOBAYASHI, T., UEDA, H., YAMAUCHI, E., WATANABE, S. AND OKUYAMA, H. (1994). Lysophosphoinositide-specific phospholipase C in rat brain synaptic plasma membranes. *Neurochemical Research* **19**, 399–406.

TUME, R.K. (1991). Characterization of polyclonal antibodies to cell-surface antigens from ovine adipose tissue. *Biochemistry International* **25**, 221–232.

VAN EWIJK, W., DE KRUIF, J., GERMERAAD, W.T., BERENDES, P., ROPKE, C., PLATENBURG, P.P. AND LOGTENBERG, T. (1997). Subtractive isolation of phage-displayed single-chain antibodies to thymic stromal cells by using intact thymic fragments. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 3903–3908.

WINTER, G., GRIFFITHS, A.D., HAWKINS, R.E. AND HOOGENBOOM, H.R. (1994). Making antibodies by phage display technology. *Annual Review of Immunology* **12**, 433–455.

PART 6

Drug Delivery