1 Introduction to Protein Purification Strategies

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If the structure of a protein cannot yet be accurately predicted from its sequence of amino acid residues, how can we reach an acceptable level of detailed knowledge about its function? For the immediate future, at least, the answer must come from the hard graft of purifying target proteins to a sufficient degree and in enough quantity to allow detailed biochemical and structural analyses. If the investigator is lucky, purification may follow a well-documented protocol. More often than not, however, purification will require modification of an existing protocol or development of an entirely new one. This chapter outlines the factors to be considered in designing a purification protocol and some of the new and exciting trends in protein purification. Much of the theory and practical details of the various separation modalities are considered in later chapters.

THE CHALLENGE

The challenge of protein purification becomes self-evident when one considers the complex mixture of macromolecules present in a biological matrix such as a cell or tissue extract. This complexity is illustrated dramatically by the protein profile of human colonic epithelial cells, determined by two-dimensional gel electrophoresis, as shown in Figure 1.1. In addition to the protein of interest (target protein), several thousand other proteins with different properties are present in any given cell type (a conservative estimate is ~5000–8000), along with nonproteinaceous

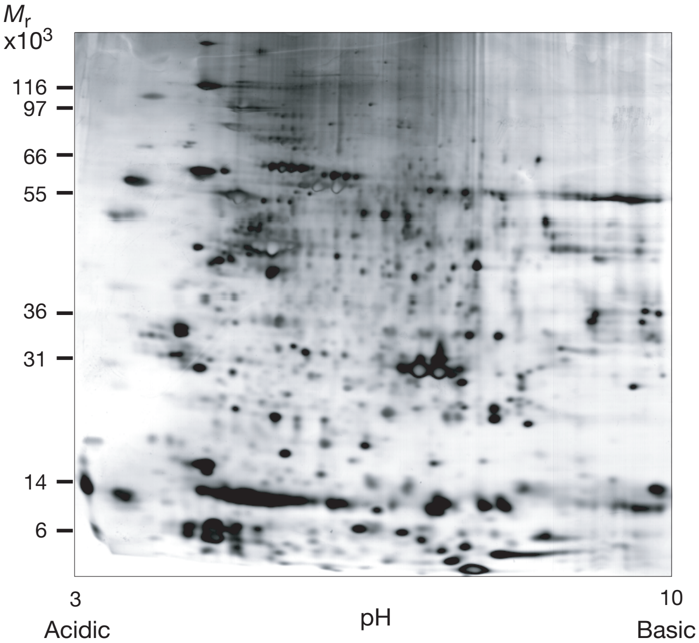


FIGURE 1.1. Human colonic epithelial cell proteins resolved on two-dimensional gels. The approximate pH and apparent molecular weight values are indicated on the *x*- and *y*-axis, respectively.

materials such as DNA, RNA, polysaccharides, and lipids. Proteins are also present within cells in varying amounts. A highly abundant cytoskeletal protein such as actin may constitute 10% of the total weight of protein in the cell extract. At the other extreme, a rare transcription factor may be expressed at such low levels (<0.001%) that only a few molecules are present in each cell. The challenge is to therefore purify (or "capture") the target protein from crude or clarified source material, free of contaminating protein, with reasonable efficiency, speed, yield, and purity.

HOW MUCH PROTEIN IS NEEDED, AND WHAT LEVEL OF PURITY IS REQUIRED?

Just how extensively a protein must be purified, as well as the scale of the purification, depends largely on the end goals, i.e., the purpose for purifying a protein in the first place. In general, one would like to obtain a target protein in a homogeneous form, free of all contaminating proteins and other materials. However, there are many applications where less than completely pure protein will suffice. The scale of protein purification depends on the amount of material required to perform a particular task. A brief outline of the quantities of protein and degree of purity required for many protein applications is given in Table 1.1.

Normal laboratory-scale biochemical purification, using conventional chromatography equipment, will typically produce 1–100 mg of protein. Microscale purification, which produces nanograms to micrograms of protein, requires chromatographic equipment specifically designed for the purpose, in particular, liquid chromatographs capable of delivering low flow rates and accurate elution gradients (Simpson and Nice 1989; Nice and Catimel 2000).

At the extreme end of the scale, if the goal is to obtain limited amino acid sequence information for the purposes of identifying an unknown protein, then only a few micrograms (1–5 pmoles) of highly purified material are required for "state-of-the-art" protein/peptide sequencing methods employing automated Edman degradation instruments. For an excellent review of the chemistry involved in Edman degradation (Edman 1949), an appraisal of the various instruments,

TABLE 1.1. Quantity and purity of protein required for different applications

Application	Amount Required	Purity Required	Comments
Identification	0.002–0.2 μg	high (>95%)	Amino-terminal sequence analysis of proteins using the Edman degradation procedure requires 5–10 pmoles (see Chapter 6 in Simpson 2003), whereas mass spectrometric approaches that rely on protein identification by accurate measurement of peptide masses and/or sequencing by collision-induced dissociation of peptides (e.g., tandem mass spectrometry) require 0.2–1 pmole of peptide(s) (see Chapters 7 and 8 in Simpson 2003). Both methods are destructive and the sample cannot be recovered.
Immunology polyclonal antibodies monoclonal antibodies protein microarrays	μg–mg	medium-high	Tens of micrograms of protein may be required as an immunogen during the preparation of polyclonal antisera and monoclonal antibodies (see Harlow and Lane 1999). The higher the purity of the immunogen, the greater the chance of raising an antibody response of high specificity. Depending on the state of the immunogen and the mode of immunization, the antibody may react only with the native form of the target protein, only with denatured forms, or with both. Antibodies that react with denatured forms are capable of detecting nanogram amounts of target protein in, for example, western blots and protein microarrays.
Enzymology	1–5 mg	high (>95%)	The amount of target protein required for enzymological studies depends on the sensitivity of the assay. The degree of purity required depends on the specificity of the assay and its susceptibility to interference by contaminants. Because these assays generally need to be repeated many times and are destructive, a reasonable goal might be to purify 1–5 mg of the target protein to >90% purity.
Biophysical analysis	mg–g	high (>95%)	Many of the methods used for biophysical characterization of target proteins (e.g., fluorescence, UV absorption spectroscopy, analytical ultracentrifugation, surface plasmon resonance, and CD analysis) permit recovery of the sample for further use.
Three-dimensional structure	10–20 mg	high (>95%)	X-ray crystallography requires ~1–2 mg of target protein to establish conditions for crystallization. An additional 5–10 mg is needed to grow crystals, large enough for X-ray diffraction. Preferably, protein should be of the highest purity achievable. Initial screening attempts can be made using target protein of ~80% purity. If no crystals are obtained, then higher purity is essential. NMR requires ~0.5 μmole of target protein (10 mg for a 20-kD protein) for initial spectra. Typically, $^{15}N/^{13}C$-labeled protein is required to solve the structure of proteins (5–20 kD). Although prolonged exposure of crystals to X-rays during collection of diffraction data may cause radiation damage to the protein, both NMR and X-ray crystallography are essentially nondestructive methods and material can be recovered for other tasks.
Pharmaceutical	mg–kg	high (>99.9%)	For clinical use, pharmaceutical proteins must be free of pyrogens and bacterial endotoxins and stable upon extended storage.

and requirements for preparation of samples, see Lottspeich et al. (1994). A detailed discussion of current automated chemical amino acid sequencing procedures (both amino-terminal and carboxy-terminal methods) including a sample preparation are presented elsewhere (Chapter 6 in Simpson 2003). With careful optimization of current instrumentation, sensitivities on the order of 0.1–1 pmole are achievable using sequencing cycles of 20 minutes per residue (Henzel et al. 1999).

One of the most exciting innovations in protein chemistry during the past decade is the development in mass spectrometry (MS) of new “soft ionization” techniques, such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). These modern MS-based techniques require only a few nanograms (0.2–1 pmole) of material to identify proteins (and peptides) on the basis of peptide masses and peptide fragment ion data, which are generated by collision-associated dissociation (CAD) of peptide ions (tandem MS). One corollary of MS-based protein identification is that the criterion of protein purity required has changed profoundly, being much less stringent than that required for conventional Edman degradation. Indeed, with MS methods, it is now possible to identify proteins in complex mixtures without prior fractionation of the mixture (see Chapter 8 in Simpson 2003).

If the goal of the purification is to obtain enough enzyme for detailed physical and kinetic studies, milligram quantities of highly purified protein are necessary. Comparable quantities of target protein are required for performing biophysical studies of protein stability and kinetics of folding that involve techniques such as fluorescence (and ultraviolet [UV] absorption) spectroscopy, circular dichroism (CD), differential scanning calorimetry, analytical ultracentrifugation, surface plasmon resonance, and hydrogen exchange.

If the purpose of purifying a protein is to determine its three-dimensional structure, either by X-ray crystallography or by nuclear magnetic resonance (NMR), tens of milligrams of highly purified material may be required. The uncertainty in the amounts required to carry out a full chemical and physical analysis of a protein using these techniques is due to the variable behavior of proteins in solution, particularly at the high concentrations (8–10 mg/ml) required for analysis. Although some proteins remain in their monomeric form over a broad concentration range (which is desirable for NMR analysis), others will aggregate or even precipitate. For X-ray diffraction analysis, a further dilemma lies in what has become a primary problem for this technology—that of growing large, single crystals (McPherson 1997). Glycosylated proteins, in particular, present a major problem due to their carbohydrate-associated charge heterogeneity and the potential of the oligosaccharides to inhibit protein–protein contacts necessary for crystal lattice packing. Biochemists are now attuned to this problem and have developed the expertise to reduce this heterogeneity by treating the purified protein with exoglycosidases or, alternatively, by using mammalian expression systems (e.g., Chinese hamster ovary cells) with genetically altered carbohydrate biosynthesis pathways for the production of recombinant glycoproteins with minimal carbohydrate heterogeneity (Stanley 1989). Investigators must be careful here and not deplete the carbohydrate completely, because fully deglycosylated proteins are often difficult to solubilize, especially at the high protein concentrations required for biophysical studies. Typical requirements for the crystallization of proteins are 5–10 mg of target protein (8–10 mg protein/ml) for screening studies and for crystallization. For reviews on protein crystallization methodologies, see McPherson (1990, 1997) and Shu and Bi (1997). In the case of NMR analysis of peptides and proteins, sample purity and the choice of solution conditions are critical factors. Samples need to be chemically homogeneous and free from low-molecular-weight protonated molecules, such as those used in many of the common biological buffers (e.g.,

CONVERTING MOLES TO MICROGRAMS

Protein molecular mass (daltons)	Amount of protein per nmole	Number of moles in 1 µg of protein
1,000	1 µg	1 nmole or 6 x 10^{14} molecules
10,000	10 µg	100 pmoles or 6 x 10^{13} molecules
20,000	20 µg	50 pmoles or 3 x 10^{13} molecules
50,000	50 µg	20 pmoles or 1.2 x 10^{13} molecules
100,000	100 µg	10 pmoles or 6 x 10^{12} molecules
200,000	200 µg	5 pmoles or 3 x 10^{12} molecules

One mole of a protein is the amount that contains 6.023 x 10^{23} molecules of that protein, which is known as Avogadro's number. The weight of a mole of a protein in grams (g) is the same as its molecular mass. For example, for a protein with a molecular mass of 20,000 daltons, the weight of 1 mole of the protein is 20,000 g.

1 mmole = 10^{-3} moles
1 µmole = 10^{-6} moles
1 nmole = 10^{-9} moles
1 pmole = 10^{-12} moles
1 fmole = 10^{-15} moles

Tris, MOPS, and TEA). A typical solvent used in NMR studies is H_2O (and dimethylsulfoxide, methanol, and acetonitrile for peptides only). For a review on the practical considerations of NMR spectroscopy of peptides and proteins, see Hinds and Norton (1997).

USING RECOMBINANT PROTEINS OFTEN SIMPLIFIES THE PURIFICATION PROCESS

Another important consideration in designing a purification strategy is whether the investigator is attempting to purify a native protein from a biological matrix (e.g., a cell line, cell-conditioned medium, or tissue) and relying on a biological assay or antibody for monitoring the purification, or whether the task is to purify an overexpressed recombinant protein. Purifying rare proteins (e.g., growth factors, receptors, or transcription factors) from natural biological sources is often extremely difficult, requiring extremely large quantities of starting material and a 1–2-million-fold purification to achieve homogeneity (see Table 1.2).

In contrast, purifying overexpressed recombinant proteins in milligram to kilogram quantities (especially for pharmaceuticals) has been greatly simplified by the ability to produce target proteins containing a fusion partner (or “a purification handle”) designed to facilitate protein purification. Biotechnology companies have commercialized a remarkable variety of sophisticated fusion proteins available for biological research. The utility of fusion proteins in an increasing range of applications is examined in a series of comprehensive reviews by Makrides (1996) and Sambrook and Russell (2001).

TABLE 1.2. Examples of low-abundance proteins and peptides isolated from natural biological sources

Protein	Source	Yield (μg)	Reference
Multipotential colony-stimulating factor	pokeweed mitogen-stimulated mouse spleen-cell-conditioned medium (10 liters)	1	Cutler et al. (1985)
Human A33 antigen	human colon cancer cell lines (10^{10} cells)	2.5	Catimel et al. (1996)
Platelet-derived growth factor (PDGF)	human serum (200 liters)	180	Heldin et al. (1981)
Granulocyte-colony-stimulating growth factor (G-CSF)	mouse lung-conditioned medium (3 liters)	40	Nicola et al. (1983)
Granulocyte-macrophage colony-stimulating growth factor (GM-CSF)	mouse lung-conditioned medium (3 liters)	12	Burgess et al. (1986)
Coelenterate morphogen	sea anemone (200 kg)	20	Schaller and Bodenmuller (1981)
Peptide YY (PYY)	porcine intestine (4000 kg)	600	Tatemoto (1982)
Tumor necrosis factor (TNF)	HL60 tissue culture medium (18 liters)	20	Wang and Creasy (1985)
Murine transferrin receptor	NS-1 myeloma cells (10^{10} cells)	20	van Driel et al. (1984)
Fibroblast growth factor (FGF)	bovine brain (4 kg)	33	Gospodarowicz et al. (1984)
Transforming growth factor-β (TGF-β)	human placenta (8.8 kg)	47	Frolik et al. (1983)
Human interferon	human leukocyte-conditioned medium (10 liters)	21	Rubinstein et al. (1979)
Muscarinic acetylcholine receptor	porcine cerebrum (600 g)	6	Haga and Haga (1985)
β_2-adrenergic receptor	rat liver (400 g)	2	Graziano et al. (1985)

Adapted, with permission, from Simpson and Nice (1989).

The growing use of recombinant proteins as pharmaceuticals requires extraordinarily high levels of purity (>99.99% purity in some cases) in order to remove materials that would be harmful when injected into a human patient (e.g., pyrogens and viruses).

PROTEINS CAN BE SEPARATED ON THE BASIS OF THEIR INTRINSIC PROPERTIES

Many proteins and peptides of biological interest are of very low abundance, often constituting <0.1% of the total cellular proteins (e.g., growth factors and signal-transducing molecules). Hence, the following are obvious constraints in obtaining sufficient quantities of such proteins in a homogeneous form suitable for biological testing and identification purposes:

- The large quantities of source material required (for examples, see Table 1.2).
- The availability of separation facilities (e.g., instrumentation).
- The physical constraints of chromatographic resin support capabilities (i.e., capacity and flow rates).

To fully exploit the chemical and physical properties of a target protein in designing an appropriate strategy for its purification, the following parameters for the target protein should be obtained in initial pilot studies:

- molecular weight (e.g., by SDS-PAGE, size-exclusion chromatography, or analytical ultracentrifugation)
- pI (e.g., by isoelectric focusing)
- stability with respect to pH, salt, temperature, proteases, inclusion of additives to protein solvents to maintain biological activities (e.g., detergents, thiol reagents, and metal ions).

Given that the logistical problems can be solved, the tremendous variation in physical and chemical properties among proteins can usually be exploited to design a workable purification scheme. Some of the relevant properties of proteins and how they can be used to purify proteins are described in the remainder of this section.

Exposed Amino Acid Side Chains Determine Protein Solubility

The solubility of a protein can differ markedly depending on the solvent, and different proteins in the same solvent can vary greatly from one another in their solubility. This protein-to-protein variation in solubility is due to the differences in the ratio of solvent-exposed charged (i.e., polar) and hydrophobic amino acids on protein surfaces. Parameters that influence the solubility of a protein include solvent pH, the ionic strength and nature of the buffer ions, solvent polarity, and temperature. Because it is not possible to predict with accuracy the solubility properties of proteins, much of the skill in purification comes from experience in handling proteins under a variety of conditions.

Proteins tend to precipitate differentially from aqueous solution upon the addition of neutral salts (e.g., ammonium sulfate), polymers such as polyethylene glycol, or organic solvents (e.g., ethanol or acetone). This behavior provides a simple means to concentrate proteins at high yield from large volumes with a concomitant two- to three-fold degree of purification. It is therefore very common for this technique to be used at an early stage of a purification, typically at the stage immediately following solubilization of the starting tissue, particularly when working on a moderate to large scale, where sample volumes are often large.

In some cases, a target protein is known to display an *unusual property* that can be exploited in a purification strategy. For example, incubation of a crude extract at low (or high) pH might lead to selective precipitation of the majority of proteins, which can be readily removed by centrifugation (because most proteins denature and precipitate at extremes of pH), leaving the target protein in the supernatant in a more highly purified form. The high stability of muscle adenylate kinase at pH 2 has been used to advantage during the purification of this enzyme (Heil et al. 1974).

The Size and Shape of Proteins Affect Their Movement Through Liquids and Gels

The size and shape of a protein affect its ability to move in a fluid solution. For a review of this subject and the variety of techniques available to estimate the molecular weight and shape of macromolecules, see Cantor and Schimmel (1980). Proteins vary markedly in size, ranging from a few amino acid residues (e.g., peptides) of a few hundred daltons to more than 3000 amino acids with a molecular mass in excess of 300,000 daltons. However, the molecular mass of most proteins falls in the range of 6 kD to 200 kD (see Fig. 1.1). Whatever its mass, the shape of a protein, which influences its movement through fluids, ranges from nearly spherical (globular) to markedly asymmetric.

These properties of proteins are exploited directly in the purification techniques of size-exclusion chromatography (SEC; Chapter 6). In SEC, a protein solution is passed through a column of porous beads (a large range of beads with variable, but defined, pore sizes are commercially available). The internal diameter of the pores are such that large proteins do not have access to the internal space of the bead, whereas small proteins have free access, and proteins of intermediate size have partial access. Large proteins therefore pass directly through the column, and smaller proteins are retarded. Although the capacity of this method is low, and its resolving capability is limited, SEC is useful for separating proteins with extremes in size. Because SEC supports are non-interactive, they have no trace-enrichment potential and cannot be used for the purpose of concentrating proteins.

One of the most important separation techniques in protein chemistry that exploits the size of proteins and peptides is SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (see Chapter 3). Analytical SDS-PAGE has long been used to monitor the purity and recovery of a target protein after each step in a purification protocol. However, equipment is now available that enables the method to be used in a preparative manner. In this method, proteins are denatured and fully coated with the negatively charged detergent SDS, such that they migrate in electrophoretic gels on the basis of their molecular weight. The pore size of the gels can be varied by altering the amount of cross-linking agent used. The gel has a sieving effect, so that the smallest proteins migrate most rapidly and the larger proteins migrate more slowly. The extremely high resolving power of this method forms the basis of the second dimension of separation in two-dimensional gel electrophoresis. In the first dimension of separation, electrophoresis is performed in the absence of SDS in a gel in which a pH gradient has been established. At a pH characteristic of each protein (the isoelectric point), the net charge on the molecule is zero and it ceases to migrate. During the past 15 years, methods have been developed for isolating and identifying proteins separated by two-dimensional gel electrophoresis (see Chapter 4). Currently, two-dimensional gel electrophoresis is one of the preferred methods in the burgeoning field of proteomics (i.e., the systematic analysis of cell or tissue protein profiles in normal and diseased states). Although high-resolving SDS-PAGE and two-dimensional gel electrophoresis methods are limited in capacity, and the protein is obtained in a denatured state, they provide a ready means for isolating small amounts of a target protein from a complex mixture.

Differences in the Surface Charge of Proteins Are Exploited in Ion-exchange Chromatography

The net charge on a protein is the sum of the positively and negatively charged amino acid residues, at the pH of the solvent. Proteins with a preponderance of basic amino acids (e.g., arginine, lysine, and histidine), referred to as *basic proteins*, will have a net positive charge at neutral pH. Conversely, proteins rich in acidic amino acids (e.g., aspartic acid and glutamic acid) will have an overall negative charge at neutral pH and are referred to as *acidic proteins*. The pH at which the net charge of a protein is zero is referred to as the isoelectric point (pI). Differences in surface charge can be exploited to separate proteins using ion-exchange chromatography (see Chapter 5). This method relies on a protein carrying a net charge of one sign binding to a solid chromatographic support bearing charged groups of the opposite sign. Proteins can be eluted from the chromatographic support by exchanging the proteins for buffer ions of the opposite charge. This is accomplished by developing the column with a gradient of increasing ionic strength. Each protein then elutes at a concentration of ionic species determined by the magnitude of the protein's surface charge. This technique, which is of intermediate resolution and high capacity, is highly selective and, in some cases, can resolve two proteins differing in only one charge.

Ligand-binding Proteins May Be Purified by Affinity Chromatography

Most proteins exert their biological function by specifically interacting with some other cellular component. For example, enzymes bind effector molecules such as cofactors, substrates, activators, inhibitors, and metal ions (e.g., Cu^{2+}, Zn^{2+}, Mg^{2+}, and Co^{2+}); hormones bind to receptors; transcription factors bind to nuclear and cytoplasmic locations, export signals, and DNA templates. These specific binding phenomena can be exploited by binding the target protein to a chromatographic column carrying the appropriate ligand or metal ion. Elution is achieved by varying the solvent conditions or introducing a solute that competes for the binding of target protein to the ligand. Various types of chromatography based on protein surface recognition or affinity, generically termed affinity chromatography, are described in Chapter 8.

The unique topology of a protein can be exploited for purposes of purification if an antibody is available that recognizes a specific determinant or epitope on the surface of that protein. Such a determinant may comprise a linear array of amino acids (linear determinant) or, alternatively, amino acids that are distributed widely in the polypeptide chain, but come into close spatial proximity upon folding of the native protein (conformational determinant). The exquisite specificity of antibody–antigen interactions forms the basis of immunoaffinity chromatography where a monospecific antibody affixed to a column selectively captures the protein of interest (see Chapter 8).

In recent years, a number of nonbiospecific affinity adsorbents have been developed. These are materials that do not interact with a target protein through a physiological ligand-binding site, but rather with other parts of the protein surface (Scopes 1987; Lowe et al. 1992). Principal among these are (1) the triazine dye ligands (Turner 1981; Qadri 1985) that have been found, by trial and error, to be highly specific for certain proteins and (2) metal atoms attached to a chromatographic support (immobilized metal affinity chromatography, IMAC) (Sulkowski 1985; see Chapter 9). IMAC takes advantage of amino acids such as histidine that can act as electron donors and thereby chelate a metal ion, thus preferentially detaining the target protein on the IMAC column. IMAC using chelated nickel has gained popular acceptance as a method to purify recombinant proteins engineered with a hexahistidine tag at either their amino or carboxyl terminus.

In general, affinity chromatography methods have high resolving capability, but low capacity, and are thus usually reserved for a late stage in a purification protocol. The exception is affinity-based purification of recombinant proteins containing an immunological or other type of affinity "tag" specifically designed to facilitate their purification.

Posttranslational Modifications Provide Additional Opportunities for Purification by Affinity Chromatography

Posttranslational modifications are fundamental to processes controlling cellular behavior, including cell signaling, growth, and transformation (Han and Martinage 1992; Parekh and Rohlff 1997). Many proteins are modified posttranslationally by the addition of carbohydrates to form glycoproteins, phosphates to form phosphoproteins, and lipids to form lipoproteins. For reviews of posttranslational modifications of proteins, see Krishna and Wold (1993, 1997). In many cases, these posttranslational modifications provide recognition "handles" that can be used in protein fractionation. For example, glycoproteins can be captured from mixtures of proteins by binding them to columns containing immobilized lectins, which are a class of plant proteins capable of selectively binding to particular carbohydrates (see Chapter 8) (Yan and Grinnell 1989; Han and Martinage 1993).

Similarly, many types of phosphoproteins can be captured from complex protein mixtures by binding to a column containing immobilized antibodies directed against phosphotyrosine or, alternatively, using IMAC (see Chapter 9).

Thermostable Proteins Can Often Be Purified Easily

Proteins are typically inactivated and precipitate if heated to 95°C. However, some proteins exhibit a remarkable degree of thermoresistance. Under these circumstances, substantial purification of a target protein can be accomplished by heating a crude extract at a temperature where the target protein is stable (active and soluble), but extraneous proteins are denatured and precipitate from solution. Examples of a purification strategy involving thermostability are those of stathmin, a key mammalian intracellular regulatory protein (Koppel et al. 1990), muscle phosphatase inhibitor-1 (Nimmo and Cohen 1978), and that of *Escherichia coli* alkaline phosphatase. *E. coli* alkaline phosphatase has an additional unusual property, an innate resistance to digestion with proteases. For example, after heat treatment of an *E. coli* cellular extract and removal of the precipitated proteins by centrifugation, the supernatant containing active alkaline phosphatase can be treated with a protease to digest the remaining soluble contaminating proteins, thereby yielding highly purified and active alkaline phosphatase.

DEVISING STRATEGIES FOR PROTEIN PURIFICATION

Before attempting to design a purification scheme, it is always worthwhile to carry out pilot experiments on the crude extract to determine whether the target protein possesses any unusual chemical and physical properties that might be exploited in a purification strategy. Useful information includes approximate molecular weight and pI; degree of hydrophobicity; presence of carbohydrate (glycoprotein); phosphate modification; free sulfhydryl groups; stability with respect to pH, salt, temperature, proteolytic degradation, and mechanical shear; and bioaffinity for heavy metals. If the nucleotide sequence is known, much of this information might be obtained by close inspection of the deduced amino acid sequence; otherwise, it can be obtained from pilot experiments using crude extracts.

Is Retention of Biological Activity Essential?

An important consideration is whether it is essential to retain biological activity of the target protein during purification. Most proteins retain activity at low temperatures in neutral aqueous buffers containing stabilizing additives such as glycerol and detergents. These conditions are incompatible with techniques in which the chromatography conditions are somewhat harsh. For example, RP-HPLC (see Chapter 7) requires the use of organic solvents (e.g., acetonitrile) and ion-pairing acids (e.g., trifluoroacetic acid) to elute proteins from the reversed-phase column. Very few proteins are able to retain their biological activity in the face of such abuse, although there are notable exceptions, such as growth factors. Similarly, the conditions required to elute proteins from immunoaffinity columns are often severe, due to the high binding affinity of antibody/antigen complexes, which dissociate only at extremes of pH (e.g., 10 mM HCl, 10 mM NaOH) or in high concentrations of salt (e.g., 3 M $MgCl_2$) (see Chapter 8).

Unless the target protein has special surface recognition characteristics that can be exploited, for example, biospecific affinity for ligands or nonspecific affinity for triazine dyes, ion-exchange chromatography, SEC, and HIC are the methods that offer the best chance of retaining biological activity. To limit the losses of biological activity of labile target proteins during purification, it is important to minimize the number of steps in the purification protocol and, where possible, avoid the need for buffer exchange between steps. It is also important to discriminate between losses of biological activity due to denaturation and physical losses caused by irreversible adsorption to the chromatographic support or by proteolytic degradation. Finally, a robust biological assay that is sensitive, and can handle a large number of samples with a rapid turnaround, is an essential requirement of a successful protein purification protocol.

Where retention of biological activity of the target protein is not required (but where the progress of purification can be monitored by western blot analysis or biosensor analysis; Nice and Catimel 1999), there is no restriction on the purification techniques that can be brought to bear on the problem. Foremost among these, especially when the end goal is identification by amino acid sequence analysis, is the high-resolving method of SDS-PAGE (see Chapters 3 and 4), followed by characterization and identification by Edman degradation or methods based on mass spectrometry (see Chapters 6 and 8 in Simpson 2003).

How Many Purification Steps Are Necessary?

Only rarely can a protein be purified to homogeneity in a single step, even when this step is based on an exquisitely specific biological characteristic. According to an analysis of 100 papers describing a successful protein purification, the average number of steps necessary to purify proteins to homogeneity is four, with an overall yield of 28% and a purification factor of 6380, corresponding to an average ninefold purification and 73% yield per step (Bonnerjea et al. 1986). In addition to the purification steps, there is often a need to concentrate and/or clarify the initial cell lysate or tissue extract and, sometimes, to include procedures for exchange of buffers between purification steps. The four generic steps of a typical protein purification protocol are illustrated in Figure 1.2.

It is generally recognized that with most conventional chromatographic supports (packings), there are compromises among speed, resolution, recovery, and capacity. Typically, this relationship is depicted as a trigonal pyramid with each apex labeled with one of these parameters (Fig. 1.3). In practice, there is interdependence between these parameters—for example, an increase in speed of a chromatographic step is usually at the expense of resolution or capacity, or an increase in resolution or capacity is usually achieved at the expense of speed. A list of procedures for fractionat-

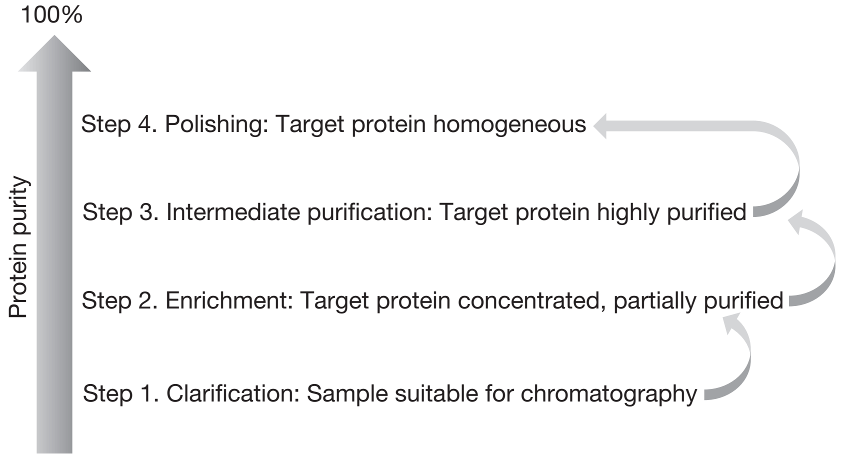

100%
Protein purity
Step 4. Polishing: Target protein homogeneous
Step 3. Intermediate purification: Target protein highly purified
Step 2. Enrichment: Target protein concentrated, partially purified
Step 1. Clarification: Sample suitable for chromatography

FIGURE 1.2. Successive stages in a typical purification protocol. Most protein purification schemes consist of at least four stages: a preliminary clarification stage, an initial enrichment (or "capture") stage, the intermediate purification, and the final polishing stage to yield a homogeneous target protein.

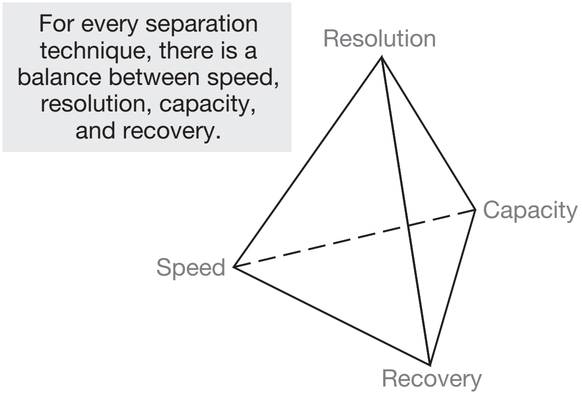
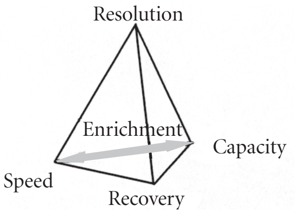
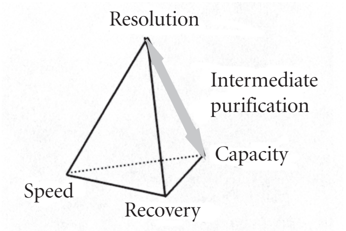
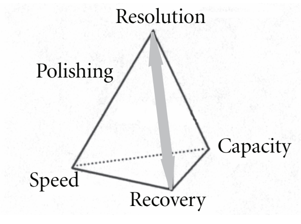
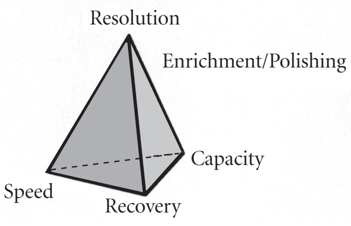

For every separation technique, there is a balance between speed, resolution, capacity, and recovery.
Resolution
Capacity
Speed
Recovery

FIGURE 1.3. Model depicting the interrelationships between important chromatographic parameters.

TABLE 1.3. Procedures for fractionating proteins and peptides

Purification Stage	Basis of Separation	
NATIVE PROTEINS		
Enrichment (capture) stage: Rapid/high-capacity, low-resolution modes		
Chromatography		
ion-exchange	charge	
hydrophobic interaction	hydrophobicity	
affinity - DNA	DNA binding	
- dye	specific dye-binding affinity	
- substrate	ligand binding site	
- lectin	carbohydrate content and type	
- immobilized metal affinity (IMAC)	metal binding	
- immunoaffinity	specific antigenic site	
Intermediate purification stage: High-capacity, low-resolution modes		 Resolution Intermediate purification Capacity Speed Recovery
Chromatography		
ion-exchange	charge	
hydrophobic interaction	hydrophobicity	
size exclusion	size, shape	
chromatofocusing	pI	
Electrophoresis		
gel-electrophoresis (preparative gels)	charge, size, shape	
Final polishing stage: Low-capacity, high-resolution modes		
Chromatography		
reversed-phase HPLC	hydrophobicity, size	
size exclusion	size, shape	
Electrophoresis		
gel electrophoresis (analytical scale SDS-PAGE, 2-DE)	charge, size, shape	
isoelectric focusing	pI	
free-flow electrophoresis	charge, size, shape	
RECOMBINANT PROTEINS WITH A FUSION TAIL (OR "TAG")		
Combined enrichment/polishing purification stage: High-capacity, rapid/high-resolution modes		 Resolution Enrichment/Polishing Capacity Speed Recovery
Chromatography		
affinity - substrate	enzyme/ligand-binding site	
- lectin	carbohydrate-binding domain	
- immobilized metal affinity (IMAC) (e.g., poly[His])	metal binding	
- immunoaffinity	antigenic epitopes	
hydrophobic interaction		
- hydrophobic amino acid tails (e.g., poly[Phe])	hydrophobicity	
ion-exchange		
- charged amino acid tails (e.g., poly[Arg])	charge (or precipitation)	

ing proteins, grouped with respect to their speed, resolution, and capacity, is given in Table 1.3; this is a generalized list only, and there are many exceptions to the rule. The strategy and rationale behind the four generic stages of protein purification, as outlined in Figure 1.2, are dealt with in turn.

Step 1: Clarifying the Starting Material

In any purification protocol that includes chromatographic methods, it is always important to incorporate an initial clarification step. Most methods for obtaining a crude extract of intracellular proteins (see Chapter 2) include steps for removing insoluble residues (e.g., differential centrifugation). However, crude extracts are often turbid and contain lipid droplets, and hence, they are seldom suitable for direct loading onto chromatographic columns, due to the likelihood of causing irreparable damage (e.g., column blockage). Since differential centrifugation (including coarse filtration through a plug of glass wool or fine mesh cloth between centrifugation steps) is awkward when handling large sample volumes and seldom results in a clear extract, it is beneficial to couple the clarification step to a rapid concentration step such as fractional precipitation using salts, polymers, or organic solvents. The most common precipitating agents are shown in Table 1.4. In addition to concentrating the protein bulk in the crude extract (this is particularly important when dealing with large sample volumes), fractional precipitation can also afford a two- to eight-fold purification of the target protein (Fig. 1.4). Precipitation is less prone to interference by nonproteinaceous material than are adsorption or chromatography procedures, and the method has a very high (~80%) average yield. It is always desirable that the initial clarification/concentration step be as rapid as possible to guard against proteolytic degradation of the target protein.

In addition to fractional precipitation, ultrafiltration is widely used for the rapid and gentle concentration of large volumes of starting material (e.g., cell-conditioned medium). Since ultrafiltration membranes are available with a variety of molecular-mass cutoff limits (1000–300,000 daltons), a modest purification of the target protein is often achieved during concentration of proteins from large volumes.

Step 2: Capturing the Target Protein

Following initial clarification/concentration, it is prudent to include an enrichment step provided the sample is amenable to column chromatography. The goal is to enrich the target protein as quickly as possible to avoid the risk of losses due to proteolytic degradation and/or other modifications. For native proteins, enrichment is best accomplished using *high capacity/low resolution* chromatographic procedures (see Table 1.3) such as anion-exchange chromatography (see Chapter 5), HIC, or nonbiospecific affinity chromatography (e.g., triazine dye chromatography). Resist all temptation to use an extraordinarily high-resolving method such as immunoaffinity chromatography as the first chromatographic step. In many cases, the high cost of immunosor-

TABLE 1.4. Procedures for clarifying and concentrating large-volume protein samples

Concentration Process	Basis of Method
Precipitation	
ammonium sulfate (including "salting-out" chromatography)	differential solubility
polyethylenimine	forms insoluble complexes with acidic macromolecules (i.e., acidic proteins, DNA, RNA)
ethanol	differential solubility
Phase-partitioning (e.g., polyethylene glycol)	differential solubility
Ultrafiltration	size and shape

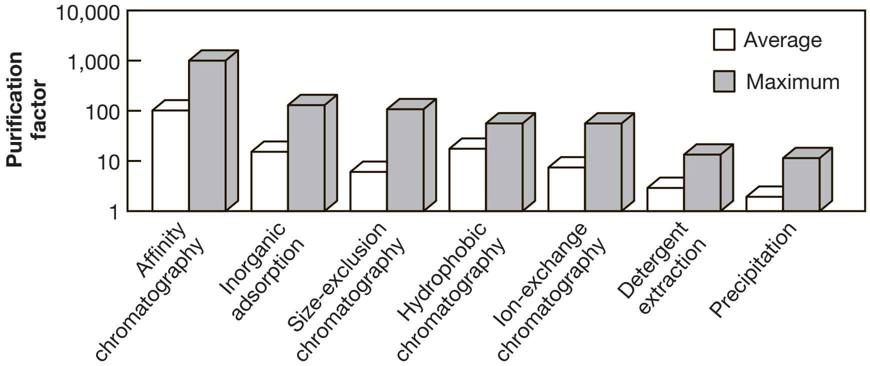


FIGURE 1.4. Average purification factors for various purification methods. (Adapted, with permission, from Bonnerjea et al. 1986.)

bents employing immobilized monoclonal antibodies prohibits the use of large columns. Repeated injections of sample therefore become necessary, which as well as being time-consuming, increase the risk of proteolytic damage to the target protein (and reduce the life of the immunosorbent). Similarly, to choose reversed-phase chromatography as the first step would be inappropriate, due to the low capacity of reversed-phase media and their lack of compatibility with subsequent purification steps, thus requiring a buffer-exchange step.

In the case of recombinant proteins, several fusion systems have been developed to promote their efficient recovery and purification from crude cell extracts or culture media (for reviews, see Ford et al. 1991; LaVallie and McCoy 1995; Makrides 1996). In these systems, a target protein is genetically engineered to contain a carboxy- or amino-terminal fusion tail (or purification “tag”) that provides the biochemical basis for some form of affinity chromatography. A variety of fusion tails have been used, including entire enzymes with affinity for immobilized substrates or inhibitors; antigenic epitopes with affinity to immobilized monoclonal antibodies; oligohistidine for recovery by IMAC; carbohydrate-binding proteins or domains recognized by lectins; and a biotin-binding domain for in vivo biotinylation, which creates affinity of the fusion protein to avidin or streptavidin. With some experimentation, fusion tails can be found that do not interfere with the biological activity of target proteins; indeed, in some cases, they have been shown to stabilize it (Ford et al. 1991). If required, a specific protease cleavage site can be engineered into the fusion tail to facilitate removal of the tail using recombinant proteases (Ford et al. 1991; Walker et al. 1994).

Step 3: Purifying and Concentrating–Intermediate Steps

This step should be designed to provide further purification (removal of extraneous protein) and reduction of sample volume (concentration), and it is best accomplished using intermediate capacity/intermediate to high-resolution chromatography (see Table 1.3). For example, SEC can be optimized to yield high resolution (see Chapter 6), but only at low speed and with small sample volumes. Likewise, immunoaffinity chromatography (see Chapter 8) becomes a viable option at this stage of the process.

Step 4: Final Polishing

The purpose of the final polishing step(s) is to remove any minor contaminants remaining (including posttranslationally modified target protein), to remove possible aggregates, and to prepare the homogeneous target protein for its immediate use or for storage. This step is best accomplished using intermediate to low-capacity/intermediate to high-resolution procedures (Table

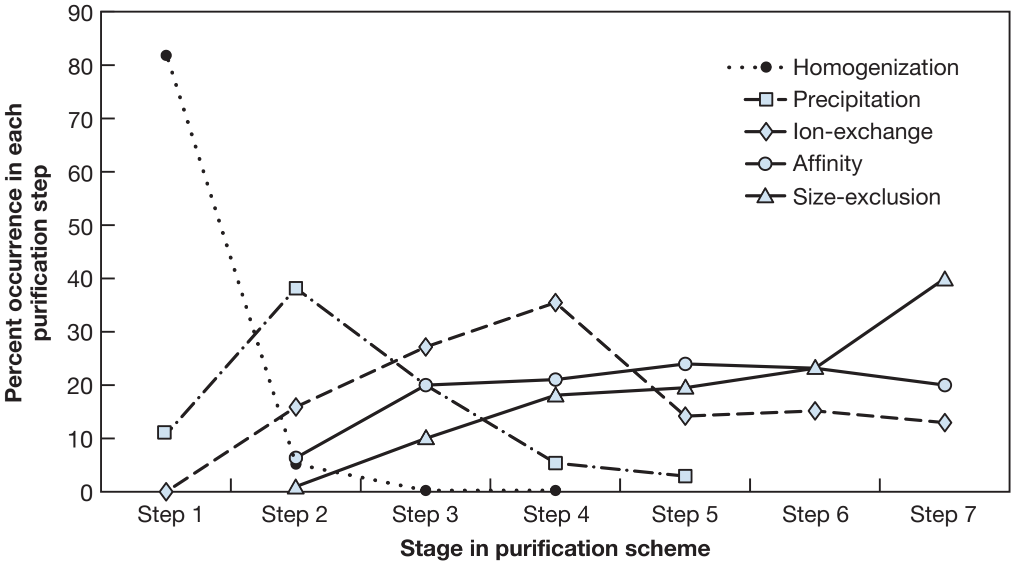


FIGURE 1.5. Order of purification steps in a generalized protein purification. (Adapted, with permission, from Bonnerjea et al. 1986.)

1.3). Although SEC has very low capacity for loaded protein, it serves an important role in removing self-aggregates of an otherwise homogeneous target protein (a necessary requirement for crystallization studies, NMR analysis, and physicochemical characterization of higher-order complexes). This step is also suitable for transfer of the protein to a volatile buffer if the protein is to be lyophilized for long-term storage.

If the target protein (especially low-molecular-weight proteins) is to be used for sequence determination and/or identification by peptide mapping of posttranslational modifications, RP-HPLC is the preferred final polishing step because of its efficiency in removing modified forms of target protein, such as proteolytic truncations and heterogeneous carbohydrate adducts. Proteins and peptides recovered from RP-HPLC columns, typically in small volumes of trifluoroacetic acid/acetonitrile, can usually be stored for long periods at –20°C (see Chapter 7; see also Chapter 5 in Simpson 2003).

Which Order of Steps Is Best?

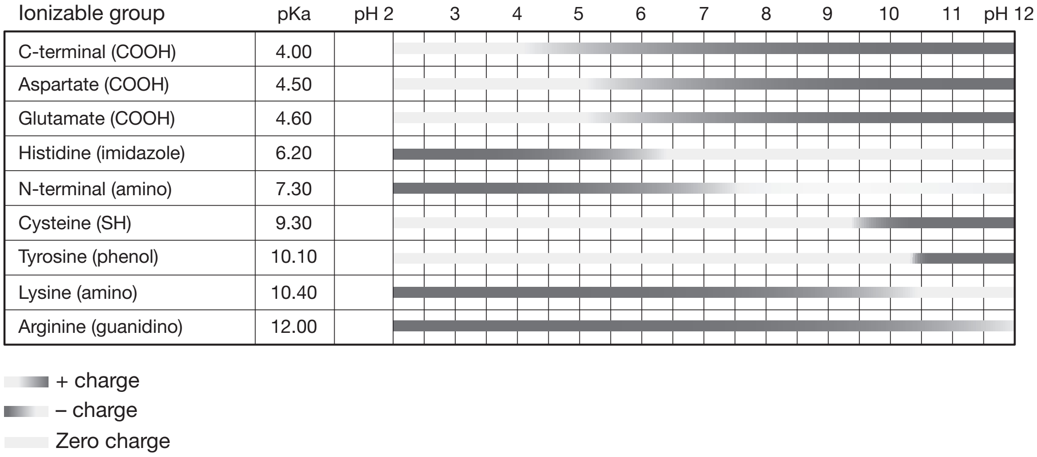
Although one would not a priori expect the sequence of steps in a purification protocol to be a major consideration, in practice, this is the case. According to an analysis of 100 successful purification methods by Bonnerjea and co-workers (1986), homogenization is generally followed by clarification/fractional precipitation, then anion-exchange chromatography, affinity separation, and finally, SEC (see Fig. 1.5). Although new methods enhance final product purity, they are typically used in addition to established procedures and not in place of them. An important consideration in designing the order of purification steps is to select, where possible, a sequence that minimizes buffer-exchange steps. For example, hydrophobic interaction chromatography (where samples are applied to the column under high salt concentrations) can follow fractional precipitation (using ammonium sulfate) or ion-exchange chromatography (where proteins are eluted with high salt concentration) without the need for a buffer-exchange step (typically accomplished by SEC, dialysis, or membrane ultrafiltration). In contrast, the use of RP-HPLC for purifying native proteins is best suited to a late stage of purification, by which time extraneous compounds that would otherwise destroy the chromatographic support have been removed.

CHECKLIST FOR PROTEIN PURIFICATION

- *Define end goals.* Decide on the level of purity and quantity required for final target protein (see Table 1.1).
- *Establish a rapid analytical assay* to monitor the purification of the target protein. Fast detection of protein activity and recovery at each stage is essential for an efficient purification procedure. The assay should also be capable of easily handling a large number of samples.
- *In pilot experiments, define the chemical and physical characteristics* of the target protein (e.g., pI, size, temperature stability, and ligand specificity) in order to simplify the separation technique selection and optimization. Where possible, use a different separation technique at each purification stage.
- *Keep the purification procedure AS SIMPLE AS POSSIBLE:* Extra steps invariably reduce the overall yield of the target protein and increase the process time.
- *Minimize sample handling at every stage* and avoid lengthy procedures that might result in reduced recovery and loss of biological activity.
- *Remove damaging contaminants,* particularly proteases, early in the purification procedure.
- *Be careful with the addition of stabilizing additives* (e.g., detergents and salts), because they may need to be removed in subsequent purification steps or they may interfere with assays.

STRATEGIES BASED ON ELECTROPHORESIS FOR SEPARATING PROTEINS

As we have seen, the reason that it is possible to separate one protein from a mixture of thousands of proteins is that a number of their physical and chemical properties vary tremendously, especially their molecular size (or weight) and charge. The latter property, overall charge, results from proteins having different numbers and sequences of amino acids, especially those that have ionizable side groups. For example, the side-chain carboxyl moieties of the acidic amino acids, aspartic acid and glutamic acid, are negatively charged at pH values greater than their pK_a values (4.4–4.6) and uncharged (un-ionized) at pH values lower than their pK_a values (see Fig. 1.6). In contrast, the side chains of the basic amino acids, histidine (imidazole group), lysine (ε-amino group), and arginine (guanidine group), are positively charged at pH values lower than their pK_a values and are uncharged at pH values higher than their pK_a values (Fig. 1.6). Because these ionization reac-



Ionizable group	pKa
C-terminal (COOH)	4.00
Aspartate (COOH)	4.50
Glutamate (COOH)	4.60
Histidine (imidazole)	6.20
N-terminal (amino)	7.30
Cysteine (SH)	9.30
Tyrosine (phenol)	10.10
Lysine (amino)	10.40
Arginine (guanidino)	12.00

FIGURE 1.6. Charge of the ionizable groups found on native proteins as a function of pH.

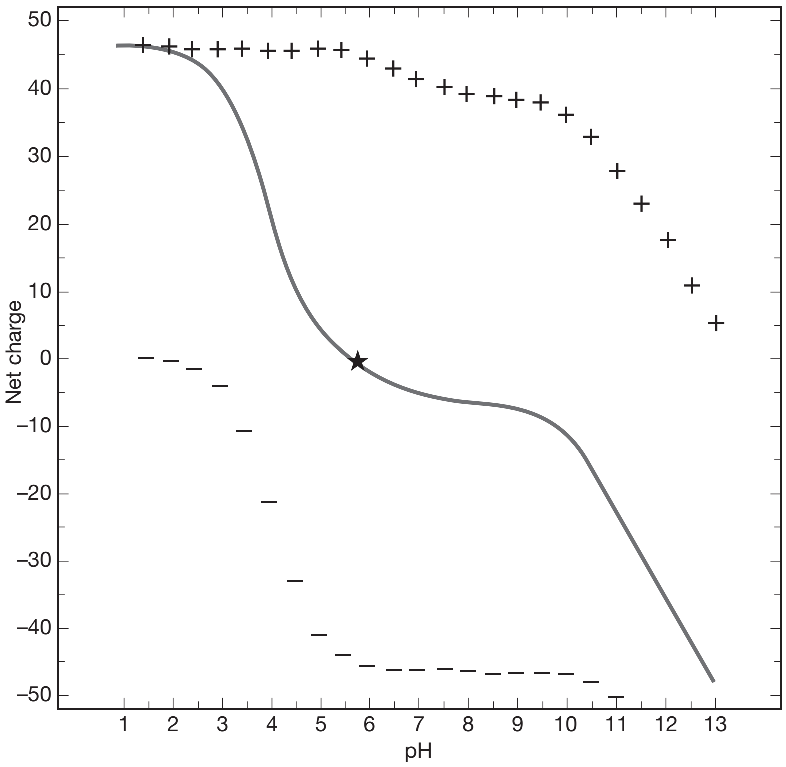


FIGURE 1.7. Titration curve and isoelectric point (pI) of *Escherichia coli* RNA polymerase transcription factor σ^{32}. Shown are theoretical plots of the number of positively charged and negatively charged amino acid side chains as a function of pH for the *E. coli* RNA polymerase transcription factor σ^{32}, based on its amino acid sequence. The pI of *E. coli* σ^{32}, indicated by the star, is 5.78. The number of each type of charged amino acids in the molecule is 23 arginines, 16 lysines, 6 histidines, 7 tyrosines, 22 glutamic acids, and 23 aspartic acids. The plot was generated using the Genetics Computer Group Sequence Analysis Software package. The usefulness of such a plot can be illustrated as follows. At pH 7.9, σ^{32} has a negative charge of 46 and a positive charge of 40, giving the molecule a net charge of –6. From this information, it is evident that σ^{32} is able to bind tightly to both anion- and cation-exchange columns, because its charge residues are not evenly distributed on the surface of the protein. Such behavior can be exploited to purify the protein, because most proteins will not bind to both types of ion exchangers under a single solvent condition. (Reproduced, with permission, from Marshak et al. 1996.)

tions are readily reversible, the proportion of charged and uncharged amino acid side groups in any protein will depend on the pH of the exogenous solution. For example, at a high pH (~12), the carboxylic acid groups tend to be charged and the side chains of the basic amino acids are uncharged. Whereas at a low pH (<4), the carboxylic groups of the acidic amino acids are uncharged and the basic amino acids are positively charged. Thus, if a protein has a preponderance of aspartic acid and glutamic acid residues, it is referred to as an “acidic protein” because it has a net negative charge at pH 7.0. Conversely, if a protein has a preponderance of basic amino acids, i.e., lysine and arginine residues, it will exhibit a net positive charge at pH 7.0. A protein’s isoelectric point (pI) is the pH at which the overall charge on a protein is zero, and it is determined by the number and titration curves of the acidic and basic amino acids on the surface of the protein (for an example of a titration and pI determination, see Fig. 1.7). Operationally, the pI of a protein is the location in an electric field at which a protein does not migrate toward either the anode (positive electrode) or the cathode (negative electrode).

Knowledge of the pI of a protein is extremely useful when designing a purification strategy, because it facilitates optimization of separation techniques such as ion-exchange chromatography (Chapter 5), chromatofocusing, and the electrophoretic separation methods outlined in Section 1 (see Chapters 3 and 4).

Enrichment of Low-Abundance Proteins by Preparative Electrophoresis

Given the dynamic range of protein abundances in biological samples such as cell lysates and blood, there is a compelling argument for subfractionation of complex protein mixtures to study low-abundance proteins. For example, the dynamic range of protein abundance in a biological sample can be as high as 10^6, with protein abundances ranging from 10 copies per cell for transcription factors up to 1,000,000 copies per cell for the cytoskeletal proteins that maintain cellular architecture.

Traditionally, 2D gel electrophoresis (Klose 1975; O'Farrell 1975) has been the preferred proteomics technique for separating hundreds to thousands of proteins in a single experiment (for reviews, see Görg et al. 2000; Rabilloud 2002; Simpson 2003). Moreover, when narrow immobilized pH gradients and extended separation distances are employed, resolution can be further improved (Wildgruber et al. 2000). Because 2D gel electrophoresis can separate only a subset of a total proteome, at best 1500–2000 proteins, this method is limited to the most abundant proteins (Gygi et al. 2000). In the small sample volumes (~10–300 µl) typically used for proteomic analysis by 2D gel electrophoresis, a large percentage of the expressed proteins are not present in sufficient amounts to be detected by current mass spectrometric methods (Gygi and Aebersold 2000; Simpson 2003). It is also difficult to resolve proteins of very high molecular weight, very low molecular weight, and very basic or very hydrophobic proteins, such as membrane-associated proteins, using this method. However, recent progress has been made in adapting 2D gel electrophoresis to high- and low-molecular-weight proteins (Tastet et al. 2003) and the judicious use of nonionic and zwitterionic detergents to solubilize membrane proteins (Luche et al. 2003; Rabilloud 2003). For the enrichment of low-abundance proteins from crude cell extracts, several protein-enriching methods have been described for separating the original protein mixture into simpler fractions each containing a lower number of proteins than the starting material. One approach takes advantage of the macromolecular architecture of a cell—the subcellular compartments, organelles, macromolecular structures, and multiprotein complexes—to break down the problem into "bite-size" components. This has led to the description of subcellular proteomes and "subproteome" analysis (for a review, see Jung et al. 2000 and articles reported in a special issue of the journal *Proteomics* [Huber 2003]). Another approach for protein enrichment involves differential detergent fractionation using detergents such as Triton X-114 (Chapter 2). Enrichment of proteins from larger volumes can also be performed by preparative polyacrylamide gel electrophoresis on the basis of protein size, usually in the presence of ionic detergents such as SDS and a preparative SDS-PAGE apparatus such as the Bio-Rad Model 491 PrepCell (Zugaro et al. 1998; Fountoulakis and Juranville 2003).

Other prefractionation methods employed to enrich fractions prior to 2D gel electrophoresis include selective precipitation, for example, with trichloroacetic acid/acetone (Görg et al. 1998), and affinity purification to isolate plasma membranes, for example, using cell surface biotinylation with affinity enrichment by immobilized streptavidin beads (Zhang et al. 2003). The chromatographic methods detailed in Section 2 can be employed in a variety of configurations to prefractionate and enrich samples for proteomic analysis.

STRATEGIES BASED ON CHROMATOGRAPHIC METHODS FOR PROTEIN AND PEPTIDE PURIFICATION

Chromatography (Greek: color writing) as we know it today is a widely used technique for separating the components of a mixture by allowing the sample (the analyte) to distribute between two phases, one of which remains stationary (stationary phase), while the other moves (mobile phase). The stationary phase may occur in many different forms including

- a packed bed of solid material in a column (column chromatography, or more commonly referred to as liquid chromatography),
- spread as a thin layer or film on a flat plate (thin-layer chromatography), or
- paper (paper chromatography).

The mobile phase may be gaseous (as in gas chromatography) or liquid. Only a liquid mobile phase is suitable for the separation of proteins and peptides. Whenever the individual components in the analyte distribute between the stationary and mobile phases to different extents, separation will occur. Compounds with a greater tendency to stay in the stationary phase will migrate through the system at a slower rate than those that favor the mobile phase.

One of the most common methods for separating proteins and peptides is column chromatography, also called liquid chromatography (LC). The stationary phase is packed into a tube or column, made of metal, plastic, or glass, through which the liquid mobile phase (the eluent, which is most often an aqueous buffer) is passed. Separation of analytes is based on their dynamic binding interaction or partitioning between the mobile phase and the surface of the stationary phase. By manipulating the chemistry of the stationary phase and/or composition of the mobile phase, along with other operating conditions (e.g., the gradient shape of eluent solvent), it is possible to obtain very subtle separations due to minor differences in molecular structure (e.g., a single amino acid modification such as an oxidized methionine or an amino acid substitution) or protein configuration (e.g., native versus unfolded structure).

Several different modes of LC allow proteins and peptides to be separated on the basis of their relative size, shape, charge, hydrophobicity, and bioaffinity. These different versions of LC are characterized by different forms of stationary phase (for a summary, see Table 1.5).

As depicted in Figure 1.8, chromatographic separation systems require the following key elements:

- ***A stationary phase*** with controlled structure (particle size, shape, etc.) and surface chemistry (bonded phase).
- ***A column*** packed with stationary phase.
- ***A mobile phase(s)*** or solvent(s) of controlled chemical composition that moves the solute through the column. For most chromatographic separations, the notable exception being size-exclusion chromatography, columns are developed using either two or three solvents that are blended in a controlled manner to produce an accurate and reproducible gradient.
- ***Chromatography equipment*** capable of accurately delivering the sample (sample injection) and controlling the blend (mobile-phase composition) and the flow of the mobile phase(s) to the column, as well as detecting the fractionated analytes.
- ***Software programs*** for blending the mobile phase(s) and running them through the column to effect the separation (i.e., procedures for developing the column).

Stationary Phase

Stationary phases (also referred to as column packings, gels, or media) are generally considered to be the key element of LC. Each is designed to provide a large surface area that is accessible to the

TABLE 1.5. Liquid chromatography methods for separating proteins and peptides

Principle of Chromatographic Separation	Type of Chromatography
Net charge	Ion-exchange chromatography (Chapter 5)
Size and shape	Size-exclusion chromatography, also referred to as gel-filtration or gel-permeation chromatography (Chapter 6)
Hydrophobicity	Hydrophobic interaction chromatography
	Reversed-phase high-performance liquid chromatography (RP-HPLC) (Chapter 7)
Biological function (bioaffinity)	Affinity chromatography (Chapter 8)
Carbohydrate content	Lectin chromatography (Chapter 8)
Antigenicity	Immunoaffinity chromatography (Chapter 8)
Metal binding	Immobilized metal ion affinity chromatography (Chapter 9)

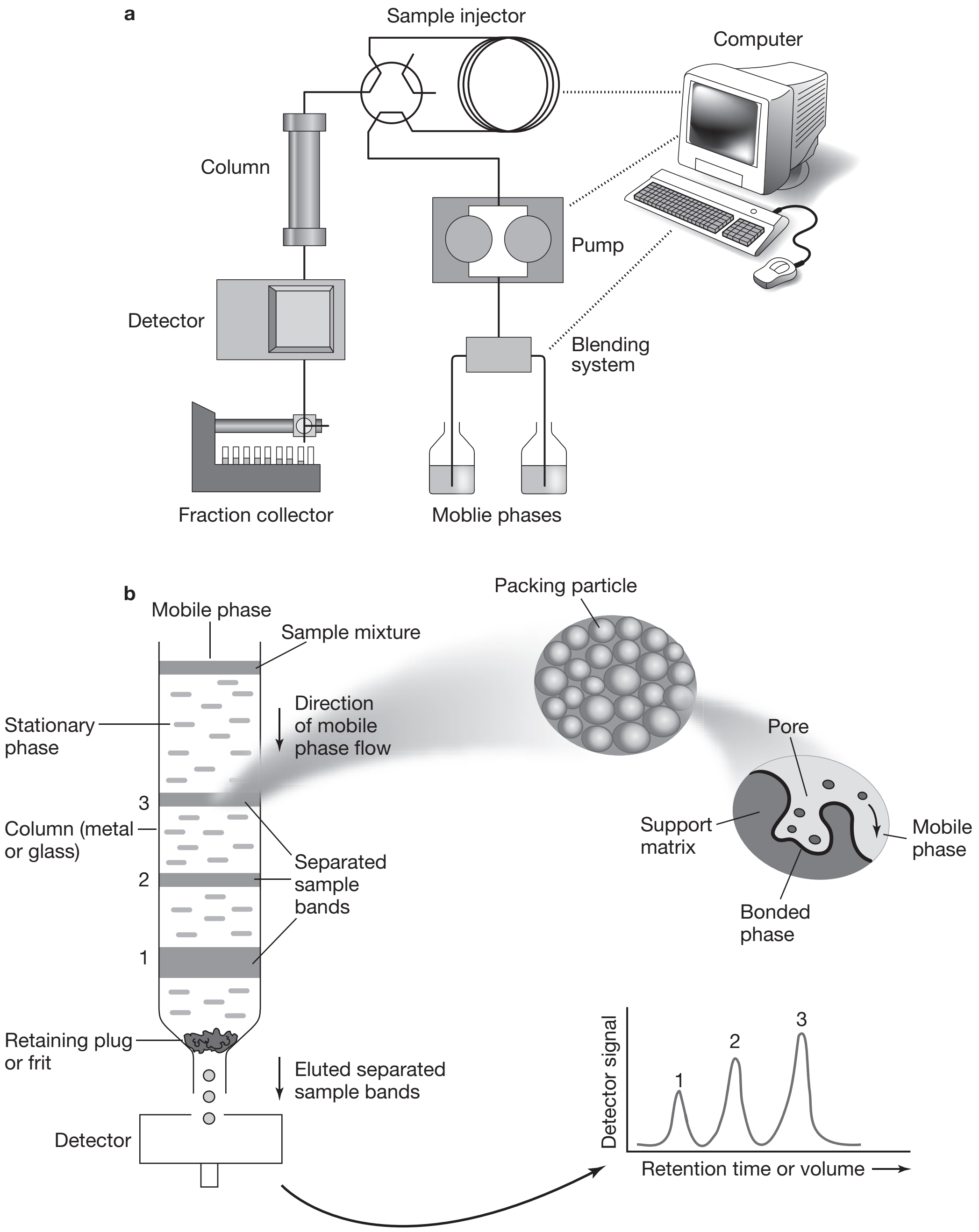


FIGURE 1.8. Basic elements of liquid chromatography. (*a*) Components of an LC system and their connections. (*b*) Details of an LC column and its packing. (Adapted, with permission, from PerSeptives Biosystems, Inc. 1996.)

mobile phase and sample molecules. This is usually accomplished by using small, highly porous particles, termed the support matrix, in which all or most of the surface area within the pores is accessible to the mobile phase. These particles form the backbone or "skeleton" of the stationary phase and provide a surface that can be chemically coated with a bonded phase containing functional groups that provide the desired specific binding interaction. Ideally, both the bonded phase and support matrix exhibit minimal nonspecific binding interactions with the analyte. Column packings for LC are classified according to the following features:

- ***Support matrix:*** rigid solids, hard gels, or soft gels.
- ***Particle size (d_p) and structure:*** spherical versus irregular particles.
- ***Pore structure:*** porous versus pellicular and superficially porous particles.
- ***Bonded phase.***

Support Matrix

A wide variety of materials have been used as chromatographic support matrices, including

- inorganic materials such as porous silica, controlled pore glass, hydroxyapatite, alumina, and zirconium;
- synthetic organic polymers such as polystyrene-divinylbenzene, polyacrylamide, polyvinyl alcohols, and polymethacrylate; and
- natural polymers such as cellulose, dextran, and agarose.

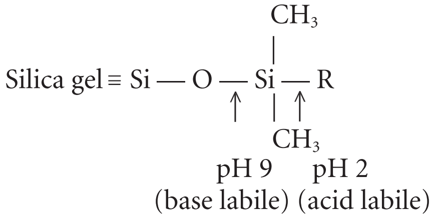
For detailed reviews describing the physical and chemical properties of various chromatographic supports, see Mikes and Coupek (1990) and Unger (1990). The basic requirements of all chromatographic support matrices are

- suitable mechanical and chemical stability,
- controlled particle and pore structure, and
- facile surface functionalization (i.e., a simple means of affixing a range of different bonded phases to the surface matrix).

In most cases, support matrices used in protein and peptide applications are hydrophilic, charge-neutral, and have low nonspecific binding characteristics. These are especially important because the bonding-phase treatment invariably fails to go to completion, leaving a small percentage of the support matrix surface without functional groups.

- ***Rigid solids*** based on a silica matrix are still the foundation of most column packings in use today. Such packings are available in a wide range of sizes, shapes, and porosity and can withstand the high pressures (4000–6000 psi) required to pack stable and efficient columns of small particles. Importantly, bonded phases with various functional groups can be readily affixed to the silica surface.
- ***Hard gels*** are generally based on highly porous particles of polystyrene cross-linked with divinylbenzene. In the early 1960s, these gels were popular for ion-exchange chromatography and size-exclusion chromatography, but were gradually replaced by rigid solids. Depending on the mode of preparation, hard gels can vary widely in both rigidity and porosity. In practice, hard gels can be used at pressures in the range of 2000–5000 psi. With the advent of high-speed chromatography using POROS resins (from PerSeptive Biosystems) (Afeyan et al. 1990) and SOURCE (from Amersham Biosciences), there has been a resurgence in the use of hard gels. Unlike conventional chromatography particles, POROS and SOURCE particles have two distinct types of pores: large pores that transect the particle and short diffusive pores that branch off from the throughpores. Compared with conventional chromatographic packings used under optimal chromatographic conditions, this design enables chromatographic separations to be carried out much faster, with little or no loss in resolution or capacity (Moritz and Simpson 1995).
- ***Soft gels*** (such as cellulose, dextran, polyamide, and other hydrophilic polymers), in contrast to the rigid gels and hard gels, cannot withstand high pressures. Soft gels are widely used for the separation of proteins based on size and shape (see Chapter 6).

It is useful to examine the structure and properties of two major types of chromatographic supports: silica and organic polymer gels. Both are manufactured as spherical beads of various sizes, often with extremely narrow size distributions. Both types of packings have been synthesized with gradations in pore size, and also as nonporous particles. The chemical resistance, especially pH stability, is a major issue regarding the utility of these supports. For instance, porous silica exhibits a somewhat pH-dependent solubility of ~100 ppm in the pH 2–8 range, but begins to dissolve rapidly in aqueous solutions above pH 9 and below pH 2. The bond between the oxygen and silicon is unstable in alkaline medium, and the bond between the silica and carbon atoms of the functional group R is unstable in a strong acid medium. This is shown schematically:



Although polystyrene-divinylbenzene-based gels are stable over a wider pH range (e.g., pH 2–12), they suffer from the slow release of monomer. In the case of dextran and agarose, both are stable toward alkaline media, but are attacked by strong acids. Both silica and the organic polymer gels exhibit matrix effects, due to parent surface-active groups that lead to nonspecific binding. Whereas the soft gels (also termed xerogels) can swell/shrink in the presence and absence of solvent, the rigid gels/hard gels (also referred to as aerogels) are rigid and their particle diameter is independent of solvent.

Particle Size and Structure

Particle size (d_p). The particle size is a critical determinant in LC, because it influences the chromatographic efficiency (and hence resolution) in a given separation. To a great extent, the distribution of particle shape and size also determines the permeability of a column and its mechanical stability (i.e., the column lifetime). It is the surface area of the bonded stationary phase and its accessibility to the mobile phase that controls analyte retention, which is reflected in the analyte capacity factor k' and the loadability of the column (see Chromatographic Performance, p. 23).

Particle shape. It is preferable to work with particles having a narrow range of particle sizes (i.e., with d_p varying no more than 1.5-fold from the smallest to largest particles), because column permeability is largely determined by the smallest particles in the column, whereas column efficiency is determined by the larger particles. Note that packed columns with very broad particle distribution are typically both inefficient and less permeable, whereas well-packed columns containing particles having a narrow range of particle sizes are both efficient and more permeable.

Chromatographic media may be either spherical or irregular in shape. Although spherical and irregular particles can each be packed to give columns of equal chromatographic efficiency, spherical materials are greatly preferred.

Recent advances in packing technology have led to the development of porous microparticles (3–20 μm in diameter d_p) that yield high-efficiency columns with fast separation times and moderate capacities and, more recently, to high-speed perfusion chromatography particles, such as the POROS and SOURCE particles. Typical particle sizes used for various LC applications are presented in Table 1.6.

TABLE 1.6. Typical particle sizes used for various LC applications

Purpose of LC	Particle Size
Analytical applications	3–10 μm diameter[a]
Preparative separations	10–40 μm diameter
Low-pressure/large-scale applications	40–150 μm diameter
Very large-scale operations	~300 μm diameter

[a] Media made from particles with a d_p <1–3 μm have proven to be impractical because of inherent problems with packing and the need for very high operating pressures.

Pore Structure (Accessible Surface Area)

Most LC packings are designed with as large a surface area as possible to provide easy access of the mobile phase and sample molecules to the bonded phase. This is accomplished using a porous resin of small particle size and with pores whose diameters approach the molecular sizes of the solutes. Typically, the pore diameter must be ~5 times the size of the molecules being purified to permit them to access all of the pores via molecular diffusion. Thus, selection of a chromatographic support with the desired porosity permits the free diffusion of analytes into and out of the pores, enabling them to optimally interact with the stationary phase. The pore structure of the packing critically affects column capacity, which is the amount of sample material that can either bind to or be separated by a chromatographic column. Since the surface area per unit volume of a particle is inversely related to the pore diameter, the use of particles with overly large pores will result in a loss of capacity—hence, both the average pore diameter and pore size distribution are critical chromatographic parameters. Chromatographic packings can be grouped according to their pore dimensions:

- Macroporous packings contain pores ranging from 1000 to 10,000 Å.
- Mesoporous packings, also referred to as "wide-pore" packings, contain pore diameters that are intermediate between the micro and macroporous resins (e.g., 180–500 Å).
- Microporous packings have 60–120-Å pore diameters.

Size-exclusion chromatography media are designed with a range of different pore diameters, which permits the separation of molecules of different sizes (see Chapter 6). Reversed-phase media, such as silica, with pore sizes of ~60 Å and 120 Å are used predominantly for small peptides, whereas the separation of large proteins—by RP-HPLC, ion-exchange chromatography, or hydrophobic interaction chromatography—requires pore diameters of >300 Å.

Bonded Phase

Whereas the column packing matrix provides the chemically inert "skeleton" for the stationary phase, the bonded phase provides the functional groups, which are designed to selectively bind solute molecules, thus separating them from the other components in the sample mixture. To minimize interference with the functional groups, the underlying packing matrix should be "neutral" and have minimal nonspecific binding with the sample.

The bonded phases of silica packings are prepared by reacting the surface silanol groups with an appropriate chlorosilane (both monofunctional and bifunctional silanes can be used for this purpose) (Fig. 1.9A). For LC particles that have polystyrene as a rigid matrix (e.g., POROS media), the bonded-phase chemistry involves first adsorbing and then cross-linking a copolymer (containing both hydrophobic and hydroxyl groups) onto the surface of the polystyrene. Once cross-linked, the hydroxyl groups are then functionalized to form the final bonded phase (Fig. 1.9B). The functional R groups are usually methyl (CH_3) groups, whereas the nature of the R′ group can be varied to give both apolar and polar stationary phases. Typically, R′ is a hydrocarbon chain (C_6, C_8, or C_{18}) that gives rise to an apolar phase (see RP-HPLC in Chapter 7). However, polarity can be introduced by substituting the terminal methyl ($-CH_3$) group in the hydrocarbon chain, for example, by a nitrile group ($-C \quad N$) or a$\overline{\overline{n}}$ amino group ($-NH_2$). For ion-exchange chromatography, the functional groups can be either acidic (cation exchangers) or basic (anion exchangers). Cation exchangers contain acidic groups that are referred to as weak (e.g., $-COO^-$) or strong (e.g., $-SO_3^-$), whereas anion exchangers contain basic groups that may be weak (e.g., $-NH_2$) or strong (e.g., $-NR_3^+$).

Several characteristics of the bonded phase critically influence chromatographic behavior, especially the selectivity and capacity of the column. Key among them are the precise chemical structure of the functional groups, the manner in which the functional groups are chemically bonded to the surface matrix surface, and the bonding density of the functional groups. Other

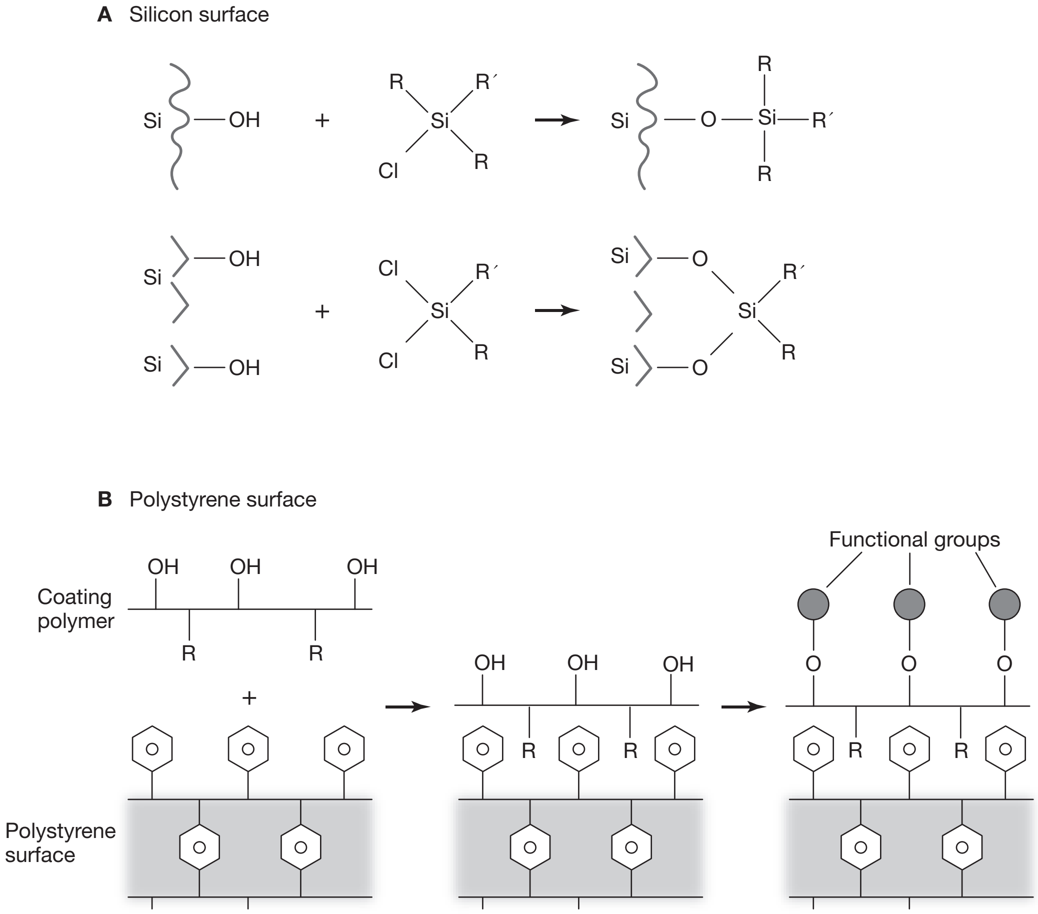


FIGURE 1.9. Bonded-phase chemistries used to affix functional groups to inert stationary-phase particles. (*A*) Solid silica particle surface. Reaction of silanol groups with monofunctional (*top*) and bifunctional silane (*lower*). (*B*) Rigid polystyrene-divinylbenzene particle surface. Bonded-phase chemistry system used with POROS media. (Redrawn, with permission, from PerSeptives Biosystems, Inc. 1996.)

important considerations are the chemical and physical stability of the bonded phase under normal operating, storage, and regeneration conditions.

CHROMATOGRAPHIC PERFORMANCE

The separation of charged molecules, such as peptides and proteins, as they move down a column is affected by (1) the differential migration of solutes and (2) their spreading or dispersion (also referred to as peak or band broadening).

Differential migration refers to the variable flow of solutes as they move down a column. Each solute partitions in a unique way between the stationary phase and the mobile phase. It is these different equilibrium distributions that cause the solutes to migrate through the column at different rates. When the interaction of a solute with the stationary phase is very strong, it is retained to a greater extent, and thus will move through the column more slowly than another solute in the sample that interacts less strongly. Such behavior can be defined by the equilibrium distribution coefficient (or partition coefficient, K_D) as $K_D = S_S/S_M$, where S_S is the concentration of a solute (S)

in the stationary phase and S_M is the concentration of the same component in the mobile phase. Individual solute bands corresponding to each component in a mixture will therefore migrate through the column at different velocities as a function of their differential migration behavior. This migration behavior is influenced by three major variables:

- Composition of the mobile phase (e.g., ionic strength, pH, and organic modifier concentration).
- Composition of the stationary phase.
- Separation temperature.

Hence, the differential migration behavior of the solute components in a mixture can be altered to improve their separation by changing any of these three variables.

Molecular spreading or band broadening is the result of dilution of a solute band as it moves down the column. It is caused by kinetic and physical processes, in contrast to the differential migration of solutes, which is driven by thermodynamic processes that arise from differences in equilibrium distribution.

Basic Retention Principles

A number of important chromatographic parameters and basic retention principles are depicted in the chromatogram shown in Figure 1.10. This chromatogram, which shows the elution profile of two pure solutes (peaks A and B), is a plot of the concentration of the two solutes. The plot is based on a spectrophotometer's response to intrinsic physical properties of the proteins or peptides, such as absorbance of peptide bonds in the UV wavelength range, amino acid side-chain absorbance in the visible wavelength range (e.g., Tyr, Trp, and Phe), or tryptophan fluorescence.

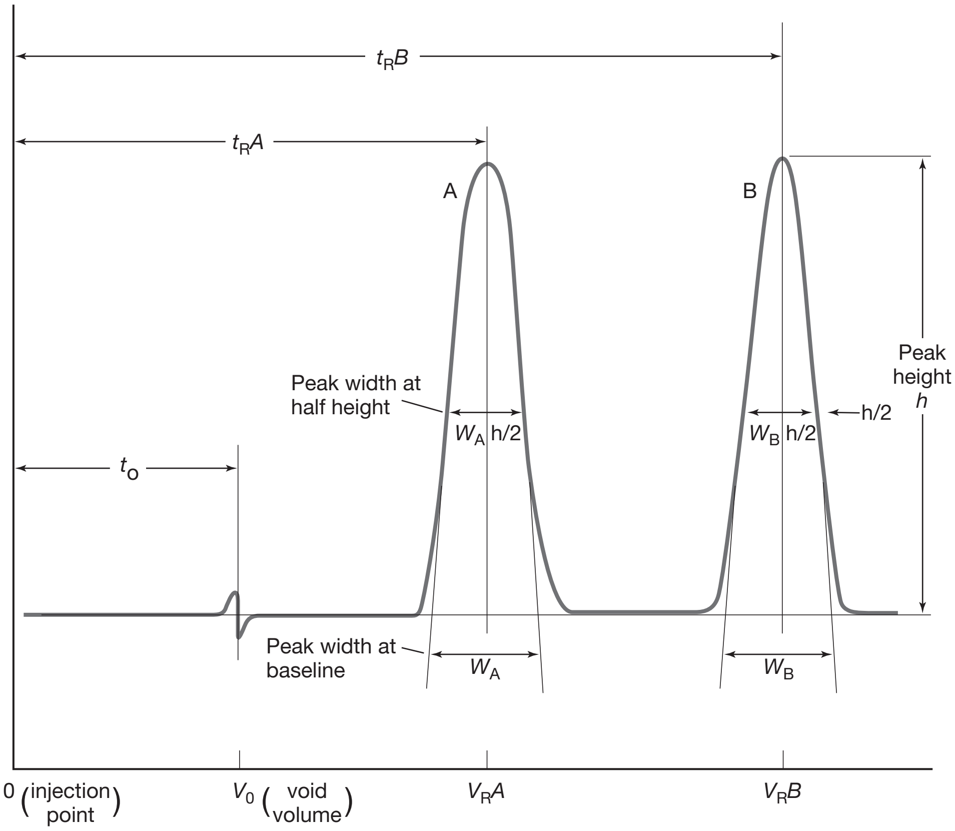
Selectivity and retention

Selectivity (called alpha or α) is a measure of the difference in retention between the solute of interest and other solutes in the sample. The retention is simply the time (t_r) or volume (v_r) it takes for a solute to move through the column, from the point of injection to the spectrophotometer, where it is monitored as an eluted peak. If a solute does not interact with the stationary phase, it will pass directly through the column and elute at time (t_o) in the void volume (v_o). The void volume is a column characteristic that represents both the interstitial volume between the particles of the stationary phase and the available volume within the particle pores. For two solutes to be resolved, they must exhibit different equilibrium distribution coefficients such that they are retained for distinct periods of time (i.e., different t_r values) and elute in different solvent volumes (v_r). The capacity factor (k')—also referred to as the retention factor—is normalized retention under isocratic elution conditions. It is a unitless term that is a measure of the retention behavior (i.e., the degree of retention) for a particular solute on a particular column, assuming equal flow rates. The capacity factor k' can be calculated by the following equation:

$$k' = (t_r - t_o)/t_o = (v_r - v_o)/v_o$$

where k' is the number of column volumes required to elute a particular solute, and t_o and v_o represent the void time and void volume, respectively. Thus, k' is directly related to the distribution coefficient (or partition coefficient) of a solute between the mobile and stationary phases (i.e., moles of solute in stationary phase per moles of solute in mobile phase) and is now well understood in both empirical and thermodynamic terms.

Selectivity is sometimes expressed as the ratio of the capacity factors k' of two solutes being separated: $\alpha = k'_2/k'_1$. In gradient elution, k' is not a valid measurement, and the simple retention time (t_r) or retention volume (v_r) is used. Selectivity is affected by the surface chemistry of the column packing, the nature and composition of the mobile phase, the nature of the stationary phase, and the gradient shape.



Retention $k' = (t_R - t_o) \ / \ t_o = (V_R - V_o) \ / \ V_o$

Selectivity $\propto \ = k'_B \ / k'_A$

Efficiency $N = 16 \ (t_R / W)^2 = 5.54 \ (t_R / W_{h/2})^2$

Resolution $R_s = (t_RB – t_RA) \ / \ (W_A + W_B)^{1/2}$

$= (t_RB – t_RA) \ / \ (W_{Ah/2} + W_{B h/2}) 0.85$

$= {}^1/_4 \ (\propto - 1) \ (N^{1/2}) \ (k'/1 + k')$

FIGURE 1.10. Important chromatographic terms and how they are measured.

Band broadening and efficiency

As solute peaks or zones migrate down a column or through a chromatography system, they usually increase in volume. This band broadening (or peak spreading) phenomenon that the solute experiences during transit through the column is an unavoidable consequence of LC, and keeping it to a minimum is a technical challenge for the researcher. The extent of band broadening that occurs during elution is reflected in the column efficiency, which is traditionally expressed in terms of the theoretical plate number N of the column: $N = 16 \ (t_r/W)^2$, where t_r is the retention

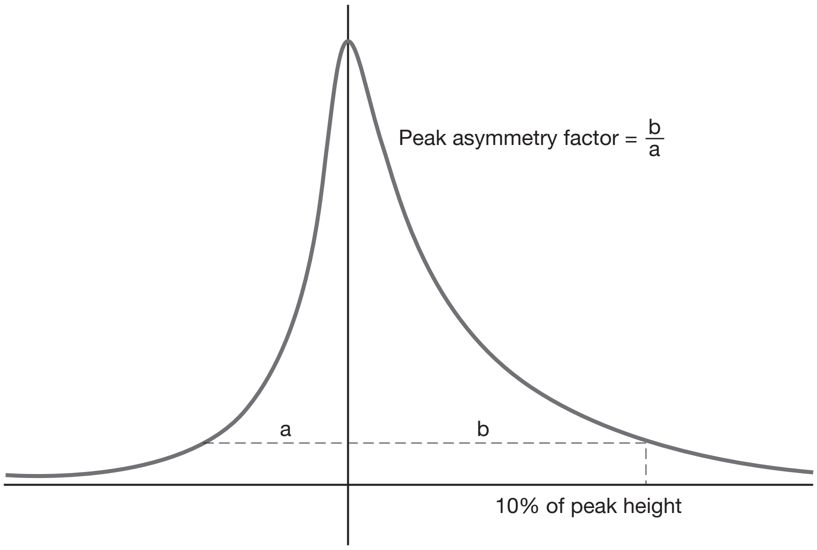


FIGURE 1.11. Asymmetric peaks. A value >1 is a tailing peak (commonly caused by sites on the packing with a stronger than normal retention of the solute). A value <1 is a fronting peak.

time and *W* is the peak width at baseline. In practice, the measurement of peak width at half height ($W_{h/2}$) has been found to be more useful (see Fig. 1.10), since it can be applied to peaks that are not completely resolved, as well as to peaks that are asymmetrical in shape (e.g., peaks that exhibit tailing; see Fig. 1.11 and asymmetry). If peak width at half height is used, then $N = 5.54\ (t_r/W_{h/2})^2$.

The value *N* is a useful measure of column performance. For a given set of operating conditions (i.e., for a particular column, mobile phase, fixed mobile-phase velocity, and operating temperature), *N* is approximately constant for different solute bands in a chromatogram. Hence, *N* is a measure of column efficiency, and, in general, the higher the number of theoretical plates, the better the column. In other words, a column with a high *N* value will provide solute peaks with narrow bandwidths (small *W* values) and improved separations. Although the value for *N* is largely independent of t_r and remains constant for different solute bands in a chromatogram, the solute peak widths increase proportionately with t_r, so that later-eluting solute bands typically show a decrease in peak height and eventually disappear into the baseline.

Since the quantity *N* is proportional to column length *L*, an increase in *L* usually results in an increase in *N* and hence a superior separation. This is especially so for LC operated under isocratic (i.e., constant-composition mobile phase) elution conditions. In gradient elution chromatography, all solute bands in the chromatogram tend to be of uniform width. The proportionality of *N* and *L* can be expressed as follows

$$N = L/H$$
$$H = HETP = L/N$$

where *H* is the height equivalent of a theoretical plate (plate height) or *HETP* and *L* is the length (usually in mm) of the column. Hence, *HETP* is a better measure of column efficiency than *N* because it permits a better comparison between columns of different lengths that are operated under identical chromatographic conditions. Thus, small values of *H* are indicative of more efficient columns. For a well-packed HPLC column of 5–µm particles, *HETP* or *H* values usually range from 0.01 to 0.03 mm.

Band broadening can also occur in parts of the chromatographic system other than the column, especially when there is excessive dead volume within the system. These extra-column contributions to band broadening can be minimized through careful attention to the type of injector used, choice of tubing, length of tubing, detector flow cell design, and dead volume of fittings, unions, and adaptors.

Resolution

Resolution (R_s) is defined as the extent of separation between two chromatographic peaks. Clearly, it is of the utmost importance, since without adequate resolution, it is impossible to achieve separation. In quantitative terms, the resolution between two peaks (*A* and *B*) can be described as the difference in retention (in either volume or time) divided by the average of the peak widths at the base of the peak.

$$R_s = \text{(difference in retention time)/(average peak base width)} = 2\ (t_{rB} - t_{rA})/(W_A + W_B)$$

A resolution of 1.0 indicates near baseline separation (overlap of only 2%), although a higher resolution (>1.5) is normally required for complete separation (Fig. 1.12).

As discussed earlier, solute retention on a chromatographic support can be rationalized in terms of both thermodynamic and kinetic considerations. Hence, resolution R_s is a composite function of both thermodynamic and kinetic parameters and is expressed in terms of an equation that includes the selectivity factor α, the capacity factor *k*′, and the plate number *N*.

$$R_s = {}^1/_4(\alpha - 1)\ (N)^{1/2}\ [1/(1 + k')]$$

From a practical point of view, it is very important to know that all three factors, σ, *k*′, and *N*, can be optimized independently with respect to resolution. The most important is selectivity, then column efficiency, and then capacity factor, which does not affect R_s to a large extent. As illustrated in Figure 1.13, for two poorly resolved solutes, an increase in the selectivity factor α results in a displacement of one solute peak relative to the other, with a profound increase in R_s. This increase in selectivity can be achieved by changing the mobile-phase pH, varying the mobile-phase solvents, altering the composition of the stationary phase and parameters such as the carbon load, or adjusting the temperature at which the separation is run.

An increase in efficiency (i.e., plate number *N*) causes a narrowing of the two solute peaks and a concomitant increase in peak height without affecting the retention times. Improving R_s by increasing *N* can be achieved by decreasing particle size, by increasing the column length, or by decreasing the mobile-phase velocity (i.e., flow rate).

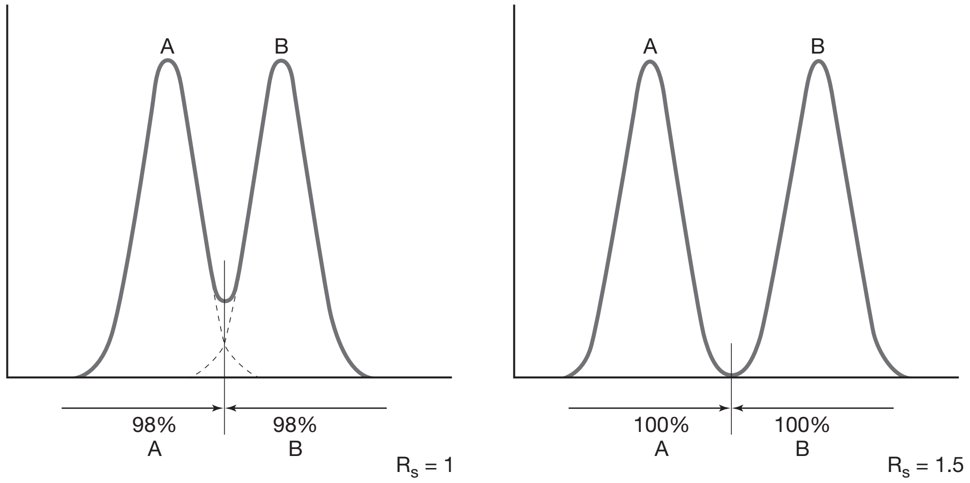


FIGURE 1.12. Separation results with different resolution. (Reprinted, with permission, from Amersham Biosciences 1999.)

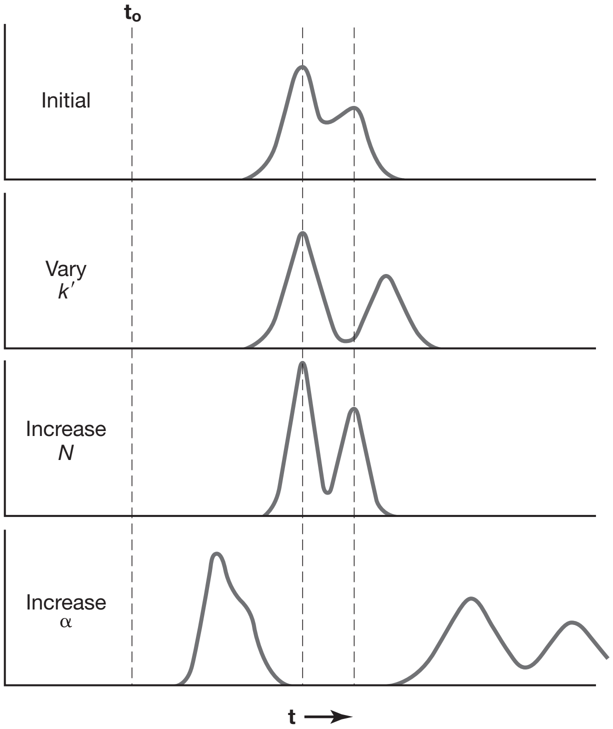


FIGURE 1.13. Effect of changes in k', N, or α on sample resolution R_s. (Reprinted, with permission, from Snyder and Kirkland 1979 [©Wiley].)

If k' for the initial separation is in the range $0.5 < k' < 2$, then a decrease in k' leads to a deterioration in the separation, whereas an increase in k' results in an improved separation. However, as k' is increased, peak heights tend to decrease with a concomitant increase in separation times (see Fig. 1.13). When k' is already within the optimal range, $1 \leq k' \leq 10$, but resolution must be improved; rather than changing the packing type, the best approach is to try increasing the N value by using a small-particle (≤10 µm) column, by increasing column length, or by decreasing the mobile-phase flow rate (at linear velocity, υ). With respect to capacity factor k' (or retention factor), resolution can be improved by changing the eluent strength.

A summary of the effects of selectivity, efficiency, and the capacity factor on resolution, and the means by which these factors can be optimized to improve resolution, is given in Table 1.7. For reviews on the general principles and basic theory of LC, see Snyder and Kirkland (1979) and Hearn (1991).

Sample Capacity

Sample capacity is the amount of sample that can be injected into a chromatographic system without overloading the column. It is often expressed as the number of grams of sample that can be bound per gram of column packing. Sample capacity is a critical parameter for preparative chromatography, because it dictates the column size and chromatographic system that are required for processing a particular sample load. In the case of analytical chromatography, sample capacity determines the dynamic range of an assay. The sample capacity can be measured in a number of ways. The following are the two most common applications:

- ***Measurement of saturation or equilibrium capacity,*** which is the maximal amount of protein that can be bound to the packing in a given mobile phase. This is accomplished by mixing a predetermined amount of packing with an excess of sample in a given mobile phase, allowing

TABLE 1.7. Effects of selectivity, efficiency, and the capacity factor on resolution

R_S	=	$^1/_4(\alpha - 1)$	x	$N^{1/2}$	x	$k' /(1 + k')$
Resolution	=	selectivity factor	x	efficiency factor	x	retention factor

Factor	Effect on R_S	How to Improve R_S
Selectivity factor ($\alpha = k_2/k_1$)	For closely spaced peaks, α is close to 1.0, so *small* changes in α have *large* effects on resulting resolution.	Alter composition of mobile phase (e.g., organic modifier pH, buffer salt), stationary phase, and/or temperature
Efficiency factor $N = 5.54\ (t_r/W_{h/2})^2$	Since R_S is a function of the square root of N, large changes in N are required to make small changes in resolution.	Increase column length, decrease particle size of column packing, or decrease flow rate. Minimize extra-column dead volume.
Retention factor ($k'=[t_r - t_o]/t_o$)	When k' is small (<1), R_S increases rapidly with an increase in k'. However, beyond a k' value of 5, R_S increases very little with further increases in k'. Separations that involve k' values >10 result in long separation times and excessive band broadening.	Alter the eluent strength. Values of k' can be increased and/or decreased by using so-called weaker and stronger solvents, respectively (see Table 7.2).

the mixture to come to equilibrium (typically, 16 hours), and then measuring the bound versus free binding molecule.

- ***Frontal adsorption analysis,*** which measures the capacity in a packed column under flowing conditions. The sample is loaded onto the column continuously until all of the binding sites on the packing are occupied (i.e., the column is completely saturated) and the sample concentration in the eluate equals the concentration being applied to the column (the latter is called the feed concentration).

Plotting the eluate concentration against the feed volume reveals the equilibrium (or saturation) capacity, which is the amount of sample that must be applied to the column to reach an eluate concentration equal to half the feed concentration (see Fig. 1.14). Another useful term,

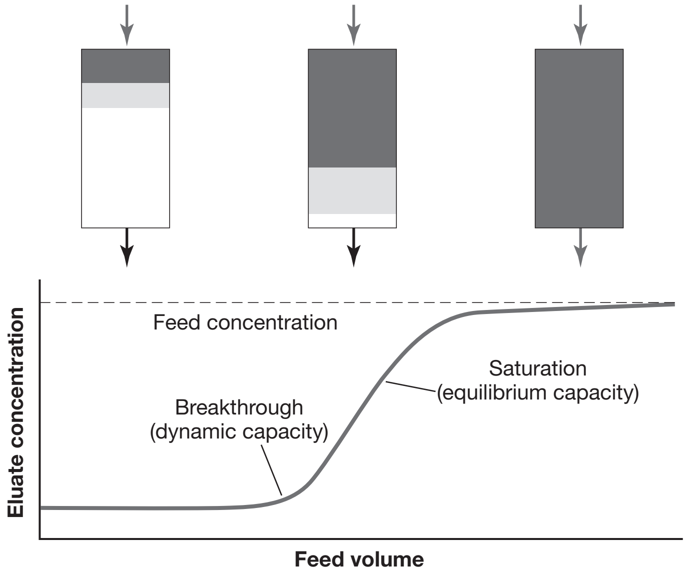


FIGURE 1.14. Frontal adsorption approach for determining the dynamic and equilibrium capacities of a column. (Adapted, with permission, from PerSeptives Biosystems, Inc. 1996.)

dynamic capacity, is the amount of sample injected onto a column in order to register the first measurable breakthrough from the column. This value is typically equal to an eluate concentration of 1%, 5%, or 10% of the feed concentration (see Fig. 1.14).

To quantitate the amount of sample in a solution by analytical chromatography, it is crucial that the amount of bound sample be linearly dependent on the concentration of sample injected. This linear range is best determined by generating an adsorption isotherm, which establishes the relationship between bound and free concentration of solute. Figure 1.15 shows that at low concentrations of sample, a linear relationship is observed between bound sample and the concentration of sample applied to the column. As the application concentration increases, the bound concentration asymptotically approaches the saturation capacity, and the column is operating in an overload range. Although this overload range is deleterious for analytical chromatography, it is the normal operating range for preparative chromatography.

For optimal chromatographic performance and to achieve the greatest resolution, column loadability is a critical parameter. Loadability can be defined as the maximum amount of sample load that can be accommodated without affecting peak bandwidth. Table 1.8 illustrates this for columns of varying internal diameters. Under optimal chromatography conditions, in which peak volumes are at a minimum, the following protein load levels can typically be achieved (Simpson and Nice 1989):

2.5–5.0 μg for a 1.0-mm I.D. column
10–20 μg for a 2.1-mm I.D. column
30–100 μg for a 4.6-mm I.D. column

Chromatography Equipment

Packing a column

The optimal procedure for packing stationary-phase particles into a column is largely determined by the nature and size of the component particles. These parameters will vary depending on whether rigid solids, hard gels, or soft gels are being considered. The column components (e.g., tubing and fittings) that are used can also have a significant effect on the choice of packing method. It is important that tubing and fittings be chosen to avoid excessive dead volumes, which contribute to extra-column band broadening. The aim in packing a column is to minimize band broadening and peak distortion. Thus, it is desirable to pack a uniform bed with no cracks or

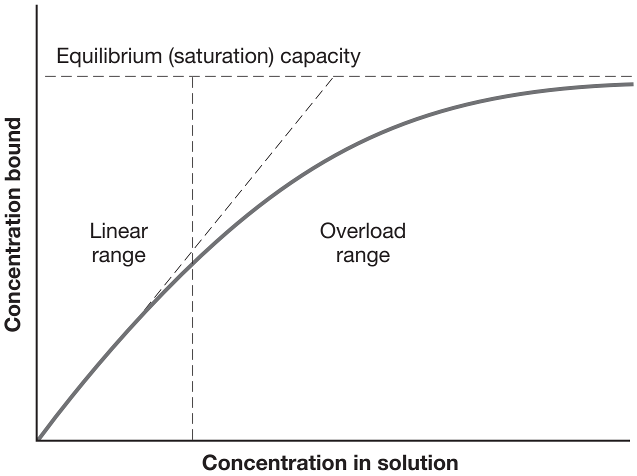


FIGURE 1.15. Representative sample adsorption isotherm depicting the equilibrium or saturation capacity and linear and “overload” regions. (Adapted, with permission, from PerSeptives Biosystems, Inc. 1996.)

TABLE 1.8. Effect of protein load on peak bandwidth for columns of varying internal diameters

Protein Load	Peak Volume (µl) for Column Dimensions			
(µg)	1.0 x 10 mm	2.1 x 3 mm	2.1 x 10 mm	4.6 x 10 mm
0.5	25	–	–	–
1.0	25	100	100	–
2.5	–	–	–	–
5.0	30	100	100	450
10.0	35	120	120	450
20	50	180	140	–
30	–	–	–	–
50	70	390	190	–
100	–	470	240	600
500	–	–	–	1200

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Support: Brownlee RP-300 (column length = 10 cm).

Protein standard: bovine α-lactalbumin.

Gradient elution (0.15% TFA to 60% CH_3CN/0.12% TFA over 60 minutes) was used at equivalent linear flow velocities: 50 µl/min, 200 µl/min, and 1.0 ml/min for the 1.0-mm, 2.1-mm, and 4.6-mm I.D. columns, respectively.

channels and to maintain a uniform particle distribution within the column, by avoiding sizing or sorting of the particles during the packing procedure. Typically, rigid solids and hard gels are packed as densely as possible under high pressure, taking care to avoid fracturing the particles during the column-packing procedure. Soft gels, on the other hand, cannot be packed under high pressure, because they compress even at very low pressure.

Optimum packing procedures for the various soft gels are described by the manufacturers (see manufacturers' Web Sites). Common procedures for packing columns with rigid solids or hard gels include:

- "Dry-fill" packing (or the "tap-fill" dry-packing procedure) for rigid particles, with d_p >20 µm.
- "Wet-fill" column-packing methods (or slurry-packing procedures) for packing particles with d_p <20 µm.

High-pressure "wet fill" or slurry-packing methods for packing high-efficiency columns were very much in vogue 20 years ago, but today they are considered to be a technically difficult art form, requiring a high-pressure slurry-packing apparatus, and hence are not recommended outside the specialist laboratory. Moreover, the cost of prepacked columns has fallen significantly in recent years, making them affordable for most laboratories. For a discussion on high-pressure slurry-packing technologies, see Snyder and Kirkland (1979).

On the other hand, many simple column hardware systems are now commercially available for packing soft gels, and the procedures for packing such columns are relatively simple. Most modern columns are of the closed type—the basic component being a cylindrical tube, usually fabricated from stainless steel, glass, or plastic. The column is fitted with two end pieces, one fixed in place and the other an adjustable adapter that allows the column length and therefore the column volume to be varied (see Fig. 1.16). The end pieces are equipped with porous frits designed to retain the packing material while still allowing the mobile phase to pass through. The frits on fixed end pieces are often made of a plastic or metal screen, whereas on the adjustable adapters, the frits are fabricated from sintered metal, glass, or plastic, and designed to minimize abrasion of the chromatographic particles and to avoid clogging of the packed column. In most cases, frits are easily exchangeable and connect to a flow distributor designed to ensure an even flow across the entire column bed; the flow distributor, in turn, is connected to the column inlet and outlet.

On some columns, both end fittings are adjustable along the length of the column and can be fixed in place with a sliding seal mechanism. To ensure good sample separation (i.e., column effi-

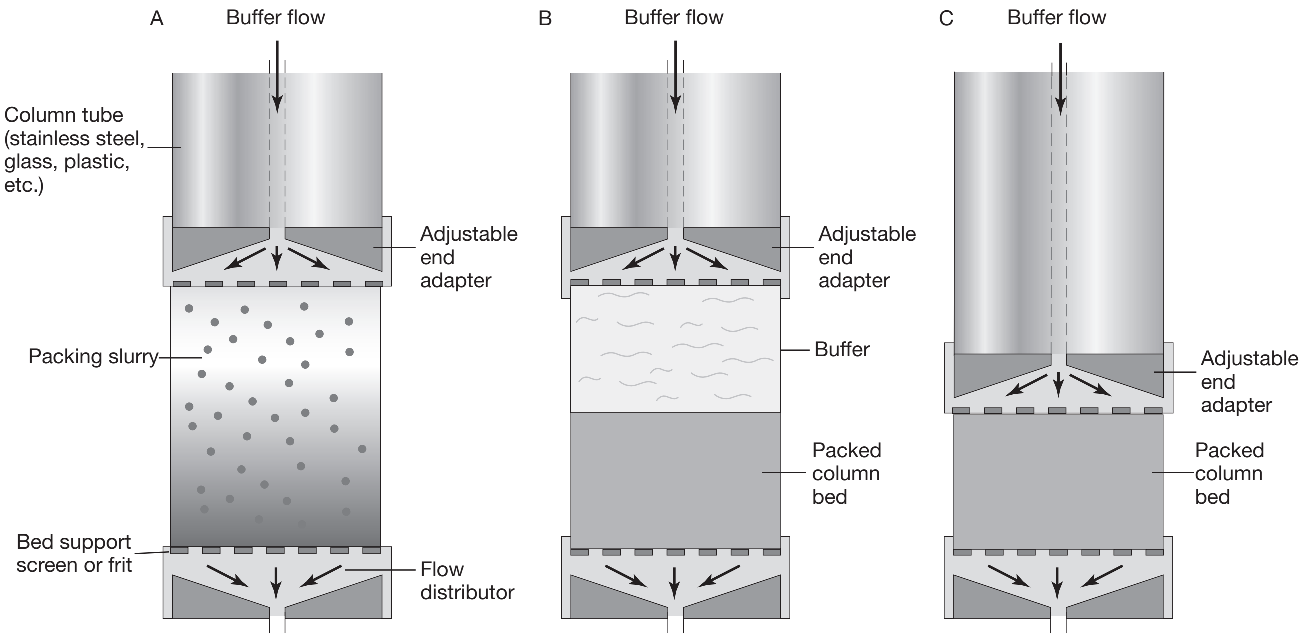

FIGURE 1.16. Basic elements of liquid chromatography column hardware and slurry packing. (*A*) Packing from the slurry settles to the bottom of the column. (*B*) Column bed is established beneath buffer. (*C*) Once column bed is consolidated, the adjustable end adapter is pushed down, so that it is flush with the top of the packed column. This eliminates any dead volume of buffer on top of the packed bed. (Adapted, with permission, from PerSeptives Biosystems, Inc. 1996.)

ciency), the column end fittings should be easily adjustable to prevent a void at the top of the bed, which is perhaps the most critical attribute contributing to poor sample resolution. If the column can be packed without any void spaces, then flow in the column can be either "up" or "down"; however, it is recommended that column flow be in the same direction in which the column was packed.

Column diameter terminology

The nature and dimensions of columns used in column chromatography can be divided into two broad categories (see Fig. 1.17).

- ***Packed columns.*** The stationary phase is affixed or bonded onto an inert solid matrix, and the particles are packed into a column. Packed columns can be further categorized based on their internal diameter (I.D.).

  Conventional columns have I.D.s >1.0 mm.

  Microbore columns have I.D.s in the range 0.5–1.0 mm.

  Capillary columns have very small I.D.s, <0.50 mm.

- ***Open tubular capillary columns.*** The stationary phase is bonded as a thin film directly onto the internal wall of a length of narrow-bore glass tubing. Two types of open tubular capillary columns are distinguished based on the manner in which the stationary phase contacts the column wall.

  Wall-coated open tubular capillary columns. The stationary phase is affixed directly to the column wall.

  Support-coated open tubular capillary columns. The walls are first coated with a layer of fine particulate material, which is then bonded with the stationary phase.

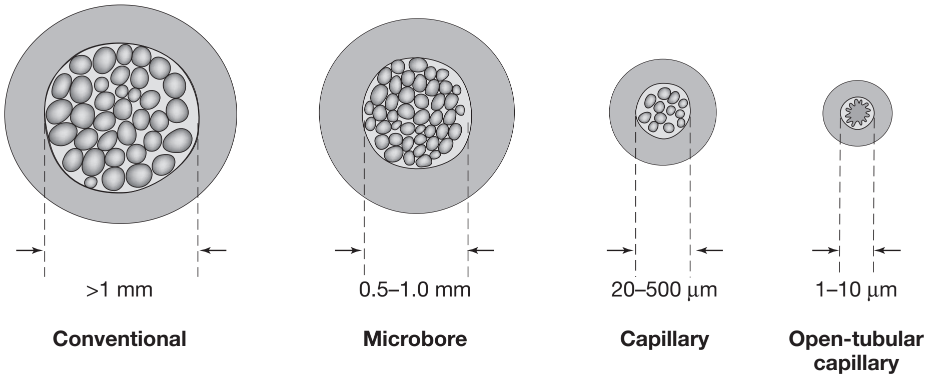

FIGURE 1.17. Columns of different diameters. The images represent cross-sections through packed columns.

STRATEGIES FOR DETECTION OF PROTEIN–PROTEIN INTERACTIONS

All the information required for the assembly and function of a cell is present within its DNA, but is realized only through the expression of the RNAs and proteins that this DNA encodes. To understand at a mechanistic level how individual cells work, how they respond to extracellular signals in the environment, and how they associate to form multicellular organisms, it is essential to comprehend the means by which proteins function in cellular organization. A critical aspect of this analysis is to define the activities of individual proteins; for example, by investigating the physical and enzymatic properties of an isolated polypeptide. However, proteins in cells do not act in isolation. Rather, their properties are controlled and modified through their interactions with neighboring polypeptides, and with other molecular components of the cell, such as nucleic acids, phospholipids, carbohydrates, and small-molecule second messengers. In this vein, the molecular machines that undertake core cellular functions such as DNA replication, transcription, and translation, are large complexes, each containing multiple proteins. Similarly, the proteins that regulate highly dynamic cellular structures, such as the cytoskeleton, or that control the transient cellular response to an external cue, or passage through the cell cycle, are also dependent on specific protein–protein interactions.When studying a particular protein, it is therefore imperative to explore the set of biomolecules with which it associates.

An extension of this concept is that multiprotein complexes, be they stable or transient, are themselves physically connected to other components of the cell. This has led to the notion that protein–protein interactions might also be exploited in the formation of larger networks that integrate multiple facets of cellular behavior. To appreciate the full complexity of cellular function, we need to know not only about localized protein interactions, but also about the connections between different protein complexes, and the properties of these more extended interaction networks (Fig. 1.18). Defining the principles that guide protein–protein interactions is a key to learning how the one-dimensional sequence of the genetic code is transformed not simply into three-dimensional protein structures, but into a versatile, dynamic, and self-propagating cell.

Identification of protein–protein interactions is a useful starting point for the delineation of cellular pathways. Often, this analysis begins with a specific subject protein of interest, and the goal is to identify proteins that were not previously known to interact with that protein. The procotols in Section 3 describe many different ways to do this. One of the oldest (but still powerful) techniques is the use of classic protein purification methods to isolate protein complexes containing the protein of interest, followed by identification of the other proteins in the complex. Chapter 11 describes the use of column chromatography to isolate complexes of interacting partners. In Chapter 14, affinity chromatography is used to select proteins fused to the glutathione *S*-trans-

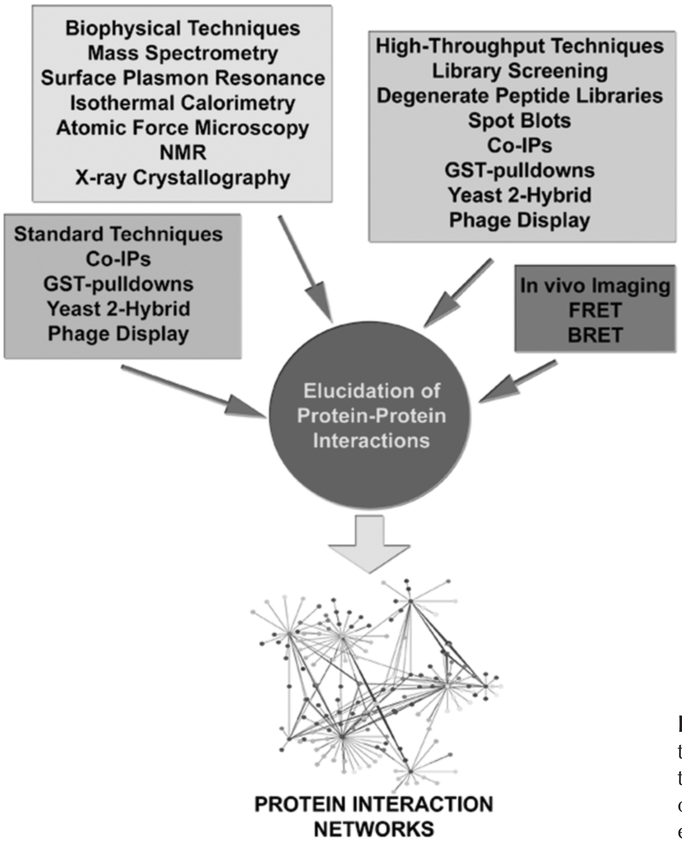

FIGURE 1.18. Flowchart illustrating how the juxtaposition biochemical and computational techniques leads to the elucidation of protein–protein interactions and the generation of protein interaction networks.

ferase (GST), which serve as probes to detect specific protein–protein interactions.

Once one or more protein–protein interactions have been identified, it is desirable to investigate the interaction(s) in detail, both in vitro and in vivo. Typically, the goals of this secondary analysis are (1) to obtain a mechanistic understanding of the proteins at the molecular level, (2) to understand the interaction's functional significance in vivo, and (3) to develop ways to specifically disrupt or perturb the interaction in vivo.

Until quite recently, the best way to determine the subcellular localization of a protein complex was by coimmunoprecipitation of the complex from a specific subcellular fraction of lysed cells (Chapter 12). In a direct extension of this approach, Chromatin ImmunoPrecipitation (ChIP) was developed as a means of localizing a DNA-binding protein or protein complex to a specific DNA sequence (Chapter 13). However, recent advances in fluorescence microscopy techniques, and the development of GFP and color variants as fusion–protein tags, have given rise to numerous other approaches to visualize protein–protein interactions in real time and in living cells. These include the use of fluorescence resonance energy transfer (FRET) and several variations of protein fragment complementation/bimolecular complementation (Chapter 15).

The genome-wide information obtained in the genomic and proteomic era has provided new opportunities that allow the identification of protein–protein interactions on a genome-wide level. In principle, it is now possible to screen every protein in the proteome for interactions with every other protein. However, the genomic/proteomic era has also provided new challenges. For example, the sometimes overwhelming body of data obtained can encourage a literal cataloging of interactions, without any reference to a specific biological question, context, mode of regulation, or any quantitation. Approaches that screen for protein–protein interactions in vivo may be best suited to addressing this problem in a single step. For example, using such methods, it should be possible to compare networks of protein–protein interactions in cells grown under different conditions. Other approaches that screen for protein–protein interactions in vivo, such as variations on bimolecular complementation (Chapter 15), can be exploited in a similar way.

At present, meta-analyses of protein interaction data, together with other functional data, indicate that no single approach is optimal. Another problem of high-throughput, genome-wide approaches is that they tend to have relatively high rates of false positives, and likely higher rates of false negatives. To overcome this, methods are being developed to compare and integrate multiple high-throughput data sets (Lee et al. 2004), including functional data sets (Bouwmeester et al. 2004; Tewari et al. 2004). By comparing two or more data sets, each obtained by a distinct approach, one can be more confident of the physiological relevance of those interactions that appear in multiple data sets. One ultimate goal of these integrated high-throughput approaches is to build a dynamic network or map of interactions that properly predicts how a complex biological system maintains a steady state, or develops and differentiates toward a specific endpoint in response to a trigger. Several analytic tools are now available, including IM Browser, Cytoscape, and Osprey. Another goal of the genomic era is to be able to predict protein–protein interactions on the basis of primary protein sequence and by referring to structures of known protein–protein interactions. Concluding the final section of the book, Chapter 16 discusses how to access and exploit the vast amount of information available in comprehensive databases. A step-by-step example illustrates how this information, together with other resources including open access and online analytical programs, allows the investigator to develop and construct a broad network of both the physical and functional interactions for a given protein.

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16 *In Silico* Tools: Analysis for Creating Focused Interaction Networks

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The preceding chapters of this book address "wet" or bench-based approaches to isolate, identify, and dissect interactions between a protein of interest and its partners. These well-validated approaches provide common points of entry into the study of new proteins of interest to a research group. Within the past 5 years, it has become possible to take a complementary approach, based on exploiting the increasingly comprehensive databases available in the post-genomic era. By combining information available in these *in silico* resources, it is now feasible to develop a relatively extensive network reflecting physical and functional interactions for any protein of interest. Most of these resources do not require specialized knowledge of computer programming, and, in fact, user-friendly programs such as Cytoscape (Shannon et al. 2003) and Osprey (Breitkreutz et al. 2003) allow researchers to generate their own local resources for their particular proteins of interest.

This chapter provides a step-by-step illustration of how to use open-access resources to develop a protein-targeted network that can be used to generate and test hypotheses (see Fig. 16.1 for an overview of the steps involved in constructing a network). For network construction, our primary tools will be protein–protein interaction (PPI) databases, canonical pathway databases, genetic interactions from model organisms, and microarray studies. We will show how these resources may be used to develop a network around a specific protein, using the pro-metastasis factor NEDD9 as our example (O'Neill et al. 2007; Singh et al. 2007). As of 2008, approximately 75 published papers cite NEDD9 as a main or peripheral topic of study. This is far fewer than the numbers of citations found for more extensively studied proteins such as Rb (>4000), BRCA1 (~6000), and ERK1 (~8000), but is typical of many proteins of current biological interest.

To view color versions of Figures 16.3, 16.4, 16.6–16.8, 16.10, 16.12, and 16.13, please see http://www.fccc.edu/research/labs/golemis/Golemis_2008/CSHL_chapter.html.

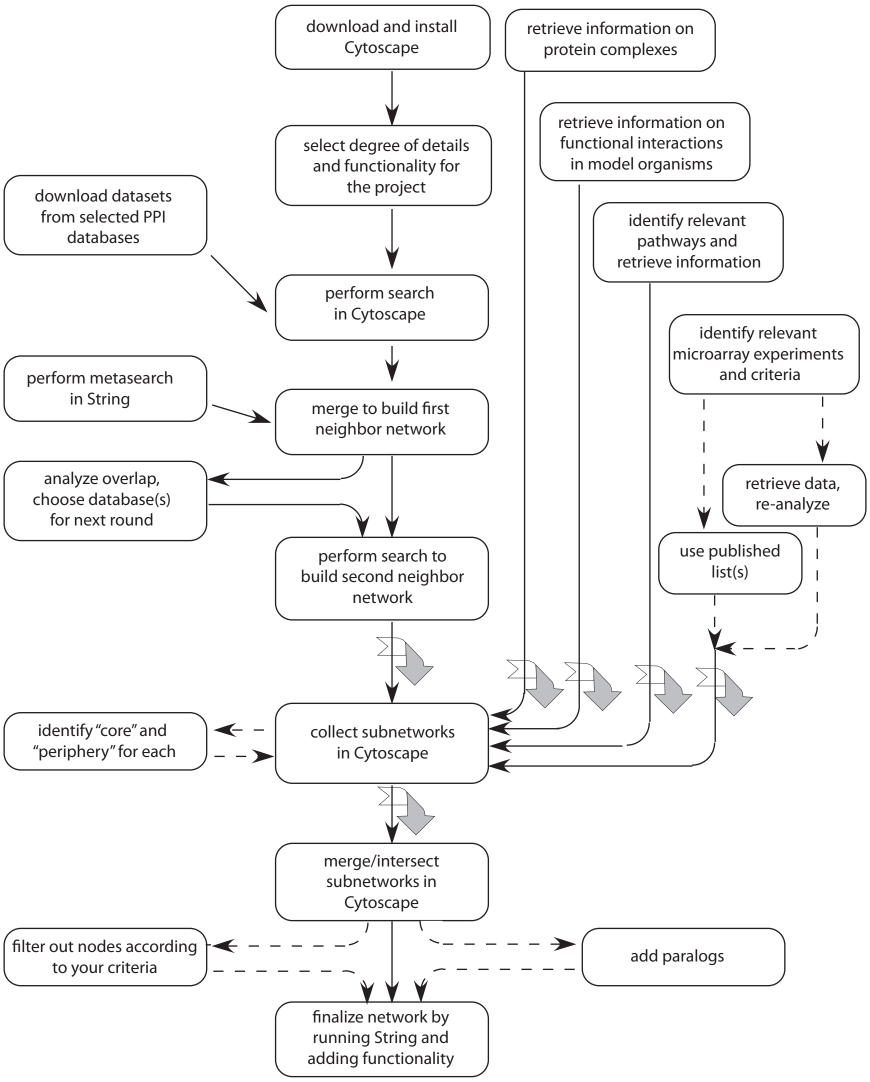

download and install Cytoscape
retrieve information on protein complexes
select degree of details and functionality for the project
retrieve information on functional interactions in model organisms
download datasets from selected PPI databases
identify relevant pathways and retrieve information
perform search in Cytoscape
identify relevant microarray experiments and criteria
perform metasearch in String
merge to build first neighbor network
analyze overlap, choose database(s) for next round
retrieve data, re-analyze
use published list(s)
perform search to build second neighbor network
identify "core" and "periphery" for each
collect subnetworks in Cytoscape
merge/intersect subnetworks in Cytoscape
filter out nodes according to your criteria
add paralogs
finalize network by running String and adding functionality

FIGURE 16.1. Flow Chart for Network Construction. Bent arrows indicate the need for ID conversion. See text for details.

Generating a network provides a useful interpretive context for direct purification experiments. The data in the network does not simply reiterate results obtained easily from direct reading of the primary literature, but provides a physical and functional interaction "landscape" that can serve to generate valuable hypotheses for subsequent studies. Finally, although this chapter emphasizes the ability to generate a network based predominantly on *in silico* resources for NEDD9, we also discuss how all of the tools and resources described here can be used with a custom set of "seed proteins" (or primary proteins of interest) derived from the application of techniques described in Chapters 1–15 of this book.

COLLECTING AND VISUALIZING DATA FOR A NETWORK

Terminology

When working with networks, we refer to individual proteins as "nodes," and the interactions between the proteins as "edges." Figure 16.2 shows a simplified network that illustrates these con-

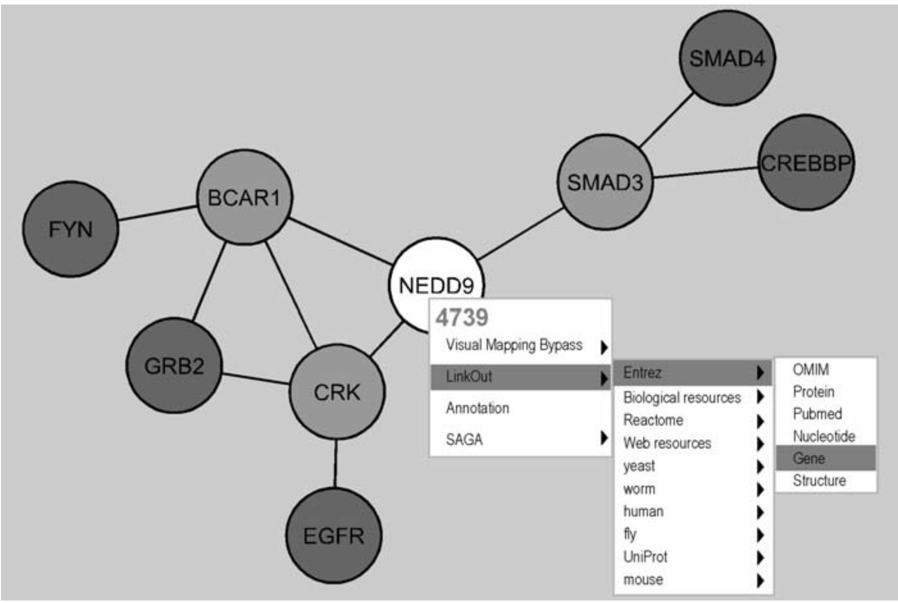


FIGURE 16.2. Nodes, Edges, and LinkOut functions in Cytoscape. See main text for details.

cepts. In the diagram, NEDD9, BCAR1, SMAD3, CRK, GRB2, FYN, EGFR, SMAD4, and CREBBP are nodes, and the lines connecting the nodes to one another are edges. All the proteins that directly interact with a specific protein are called the “first neighbors” of that protein. “Second neighbors” are all of the proteins that directly interact with the first neighbors of the specific protein. In Figure 16.2, NEDD9 is the starting protein of interest or “seed” (*white*); BCAR1, SMAD3, and CRK are the first neighbors of NEDD9 (*light gray*); and GRB2, FYN, EGFR, SMAD4, and CREBBP are the second neighbors (*dark gray*) of NEDD9, which directly interact with the first neighbors (*light gray*).

Choice of Display Tools

The first step of network assembly is to download and become familiar with the workings of one or more of several publicly available software packages for data management and display. These programs provide users with tools to visualize gene/protein interaction networks, to map experimental data back onto the network for analysis, and to facilitate analysis by providing direct links to other web-based resources.

Which program to select as the main platform for work depends on which features are regarded as most useful for a given project, as each package has some advantages and disadvantages. Questions to keep in mind include the following:

1. Is it more valuable to have ease of use, or more tools to analyze the data?
2. For visualization, how important are display aspects (e.g., multiple color tones, shading)?
3. Will it be desirable to have multiple networks open at one time?
4. For navigating among datasets, what options for selecting nodes are the most important (for example, selecting individual nodes manually; by defined properties such as gene ontology [GO] terms; by interaction confidence levels; etc.)?
5. What options for import and export of tables, Excel files, and graphics are available?
6. Can searches be done online (or do they require importation of very large files onto local computers)?

7. How important is it to be able to add customized information (i.e., specific links) or overlay additional information (such as GO categories, expression data, or cell compartmentalization)?
8. Is it important to include analytic tools to assess network topology?

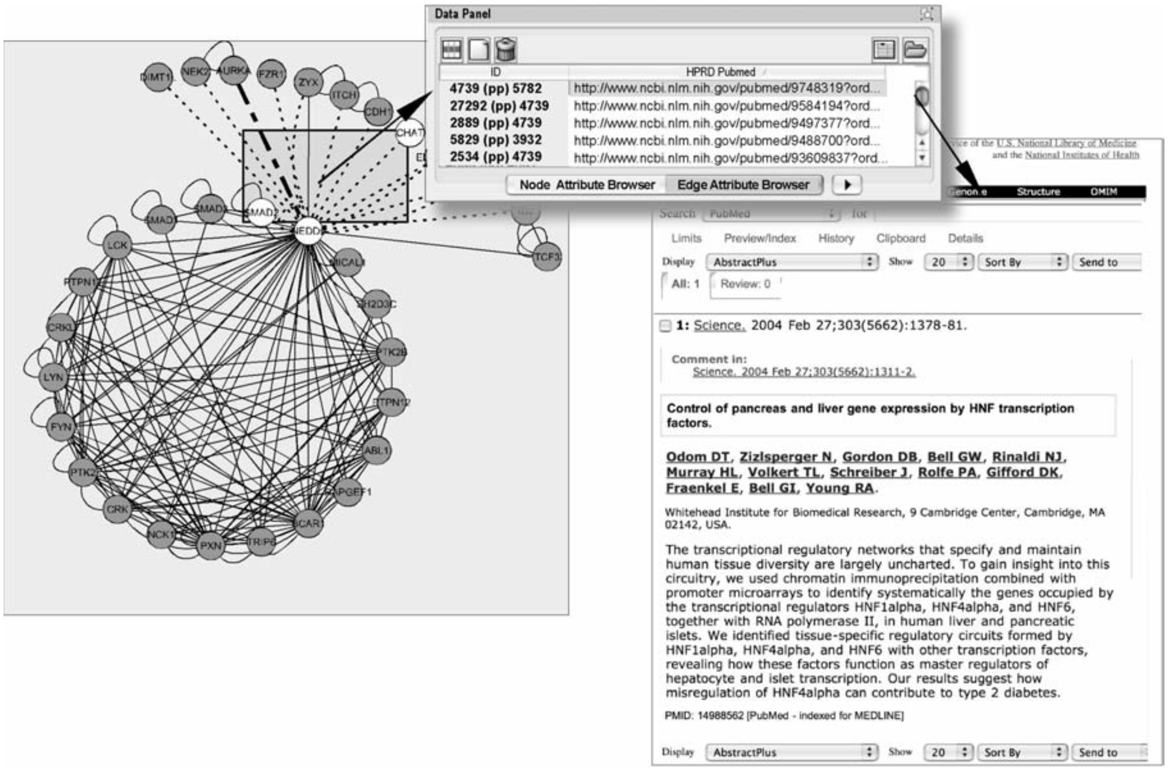
Various available programs include IM Browser (Pacifico 2006), Osprey (Breitkreutz et al. 2003), and Cytoscape (Shannon et al. 2003). Among these, IM Browser was originally developed for managing *Drosophila* data, and to date has been most used in that context. Osprey has a number of useful features: Created by professional software developers, it allows seamless import from a very useful database, BioGRID (Stark et al. 2006), and works with genes designated by their gene symbol. Comparative weaknesses of Osprey include the relative difficulty in selecting nodes in densely displayed networks, the limited number of layout options, and the relative inability to add additional features. For those starting out in network construction, Cytoscape is extremely user-friendly and is available in versions that run on both Mac and PC platforms. In contrast to Osprey, Cytoscape has been made open source by an active community of developers (www.cytoscape.org). Although this community-based development process causes some difficulties (notably in program bugs), it has generally been extremely successful in creating a powerful resource.

A detailed Cytoscape manual (that can be freely downloaded from the resource website) provides information for getting started with the program and for using its optional advanced tools. Our intention here, therefore, is not to provide precisely detailed instructions for network construction, but to discuss strategies. Cytoscape allows the user to import protein data files and export assembled networks in easily manageable file types, such as tab delimited or Excel spreadsheet files. The program also offers many interactive tools that can be used when viewing the network. For example, the LinkOut tool displays the links from a specific node to many different bioinformatics resources that provide further information about a given protein. In the example shown in Figure 16.2, the Entrez Gene option is used to retrieve the Entrez Gene webpage for NEDD9: 4739 is the Entrez ID for NEDD9. Which links are available depends on how node IDs are imported and maintained in Cytoscape. If protein lists are imported as Entrez ID numbers, only Entrez links can be used initially. However, if information from a different resource is desired, the node requires alternative annotation with the correct identifier for use with the alternative databases (tools for ID conversion are discussed in following sections).

Cytoscape also makes it easy to collect, store, and display additional information about proteins and their interactions. This information, termed node/edge attributes, can be used in visualization of the network. For example, one simple option available in Cytoscape allows the user to color nodes of specific subsets different colors (shown in Fig. 16. 2) to emphasize first (*light gray*) and second neighbors (*dark gray*). The Cytoscape package allows the automatic generation of many attributes (e.g., based on network topology or GO functions), but the user may want to add some characteristics to the network that are unique to his/her interest. In these circumstances, Cytoscape allows the importing of node and edge attributes. For example, several of the PPI databases provide PubMed IDs associated with reported interactions. Figure 16.3 shows a PubMed link to the abstract of the publication in which a specific PPI was confirmed. To incorporate this feature or characteristic, the user creates a hyperlink as an edge attribute to import into Cytoscape. The user then highlights a subset of the PPI network and views the details of the edge attributes by clicking on the "edge attribute browser." A single click on the hyperlink opens a web browser and displays the PubMed abstract, shown in the bottom right corner of Figure 16.3.

Gathering and Merging Information

Networks can combine different classes of information, including PPIs, data from "expert systems," gene and protein expression data, and genetic interactions identified in multiple model organisms. How this data is combined depends on the goals of the project, and the complexity of



Data Panel

ID	HPRD Pubmed
4739 (pp) 5782	http://www.ncbi.nlm.nih.gov/pubmed/9748319?ord...
27292 (pp) 4739	http://www.ncbi.nlm.nih.gov/pubmed/9584194?ord...
2889 (pp) 4739	http://www.ncbi.nlm.nih.gov/pubmed/9497377?ord...
5829 (pp) 3932	http://www.ncbi.nlm.nih.gov/pubmed/9488700?ord...
2534 (pp) 4739	http://www.ncbi.nlm.nih.gov/pubmed/93609837?ord...

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□ 1: Science. 2004 Feb 27;303(5662):1378-81.

Comment in:
Science. 2004 Feb 27;303(5662):1311-2.

Control of pancreas and liver gene expression by HNF transcription factors.

Odom DT, Zizlsperger N, Gordon DB, Bell GW, Rinaldi NJ, Murray HL, Volkert TL, Schreiber J, Rolfe PA, Gifford DK, Fraenkel E, Bell GI, Young RA.

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The transcriptional regulatory networks that specify and maintain human tissue diversity are largely uncharted. To gain insight into this circuitry, we used chromatin immunoprecipitation combined with promoter microarrays to identify systematically the genes occupied by the transcriptional regulators HNF1alpha, HNF4alpha, and HNF6, together with RNA polymerase II, in human liver and pancreatic islets. We identified tissue-specific regulatory circuits formed by HNF1alpha, HNF4alpha, and HNF6 with other transcription factors, revealing how these factors function as master regulators of hepatocyte and islet transcription. Our results suggest how misregulation of HNF4alpha can contribute to type 2 diabetes.

PMID: 14988562 [PubMed - indexed for MEDLINE]

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FIGURE 16.3. Retrieving information about sets of nodes. In the network window of Cytoscape, dragging a selection with the mouse arrow (*rectangle*) will select nodes and/or edges, depending on the setting selected. In the example shown here, Cytoscape marks selected nodes in white, and selected edges with dashed lines. Any information, which the researcher judges to be important or relevant to the project, can be saved in Cytoscape as an "attribute," and will be shown in the Data Panel upon selection. This attribute can be an Internet link. In this case, clicking on it in the Data Panel will open a new browser window on a corresponding web page. Cytoscape automatically highlighted the edge selected in the Data Panel with bold dashed lines. (Color images displayed at http://www.fccc.edu/research/labs/golemis/Golemis_2008/CSHL_chapter.html.)

the dataset (see Fig. 16.4). Increasingly, as databases are populated from combined detailed and high-throughput studies, it is possible to construct a very rich landscape around a protein of interest. To combine information from different resources productively, it is usually necessary to perform ID conversions for each node. Due to lack of standardization, the recording of gene information varies among the different databases; the identifying terms can range from a Gene ID (a number) to a symbol or Refseq. Sometimes the identifiers are unique to the database, but are not used as a common identifier in the scientific community. In these cases, it is necessary to use a common identifier system for all nodes in the database in order to apply tools to the entire network.

Gene/protein ID converters, such as Clone/Gene ID Converter, are very useful as a starting point for ID consolidation, and significantly expedite database construction (Alibés et al. 2007). These tools work with lists of gene IDs collected from a database and are saved as simple text or Excel files. Frequently, however, these tools cannot accurately convert all of the proteins in a list of interest: Losing nodes to ID conversion is a common problem when merging information from databases using solely automated approaches. For example, many Entrez IDs are replaced or removed on a regular basis due to advances in knowledge, making it difficult for the curators of the gene/protein ID converters to keep up-to-date and thus convert every gene/protein in a list. Hence, manual conversion for "orphan" IDs is often necessary.

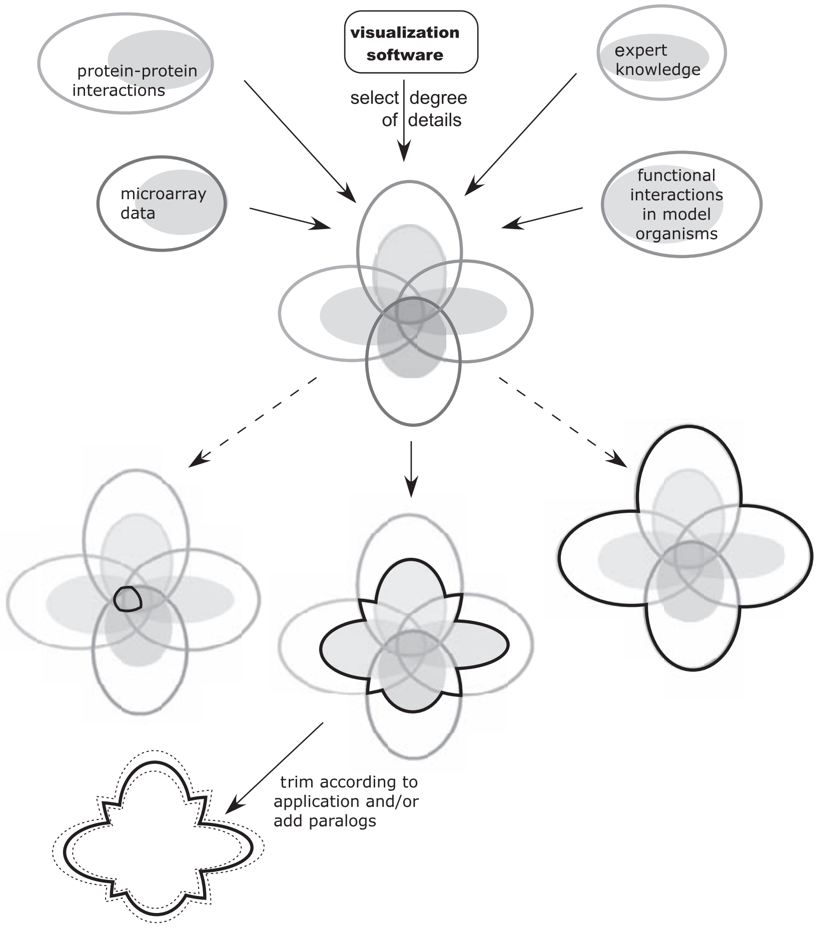

visualization software
select degree of details
protein-protein interactions
expert knowledge
microarray data
functional interactions in model organisms
trim according to application and/or add paralogs

FIGURE 16.4. Options in combining data. Once the user becomes familiar with a visualization tool, data is imported from options including PPI databases, model organism-based functional interaction databases, and co-expression (e.g. microarray) databases and pathway maps (expert knowledge). In building a resource, both "core" datasets, reflecting proteins linked to the network seed in many databases, and "context" datasets, reflecting proteins more distantly connected to the seed or found only in one or two databases, are generated. How this data is used depends on the researcher's ultimate goal. In option 1 (*left*), only data found in multiple orthogonal datasets is selected, for example. This extreme option would be useful when working with a very well-known gene, to confine the list of targeted candidates to a reasonable size for mid-throughput experiments. In option 2 (*center*), proteins found in multiple "core" datasets connecting to the seed are selected, and the intersections between the "context" datasets are added, to define an interaction sphere of high value candidates. In option 3 (*right*), all proteins linked to the seed by any of the search criteria are maintained as a resource that can be mined as needed to build context around high value proteins that emerge as linked to the seed. In this case, it may also be useful to run the merged dataset through the STRING resource to potentially retrieve additional connections between the genes, based on orthogonal datasets. (Color images displayed at http://www.fccc.edu/research/labs/golemis/Golemis_2008/CSHL_chapter.html.)

Importantly, the automated conversion system is unlikely to "swap" one gene for another, whereas an investigator working manually might be tempted to substitute commonly used names for given genes. For example, NEDD9 is known to interact with the protein CHAT/NSP3; however, the official name for this protein is SH2D3C (SH2 domain containing 3C, Nsp3), with the symbol CHAT reserved for choline acetyltransferase. Another caveat in ID conversion is that some gene symbols can signify completely unrelated genes in different species. For example, a search for the gene symbol PKC in Entrez Gene turns up *p*rotein *k*inase *C* in *Sus scrofa* (pig) and in *Apis mellifera* (honeybee) but *p*aroxysmal *k*inesigenic *c*horeoathetosis in *Homo sapiens*. The gene symbol for human protein kinase C is PRKCX, where the additional letter "X" represents the specific isoform of the gene. Hence, when assembling the initial resource, it is important to check results individually by reading gene descriptions and comparing the numbers of genes in input and output lists.

In working with human proteins that have been the topic of little or no previous formal study, the investigator may sometimes find no hits in initial database searches. In these cases, performing initial sequence-based searches to find homologs or likely homologs in other species can sometimes provide suitable "seeds" that can be used to identify near neighbors. If necessary, homology searching can be sequence-based, using standard NCBI resources such as BLAST. For most genes, the Homologene and Unigene functions readily identify likely orthologs across species.

PPI DATABASES

As shown in Figure 16.1, the identification of PPIs is a useful first step in network construction. Several of the numerous PPI databases (shown in Table 16.1) provide overlapping information. A graph comparing the numbers of interactions in each database is shown in Figure 16.5. Within each database, protein interactions are annotated with detailed information regarding the "report" of a protein interaction and the relevant experimental data. This information facilitates our making judgment calls for inclusion or exclusion of any given interaction. For the NEDD9 test case, we used the databases providing the most interactions: BIND, BioGRID, and HPRD, each of which is described below.

The Biomolecular Interaction Network Database (BIND) archives protein, RNA, DNA, small molecule, carbohydrate, and lipid interactions. The interactions in this database span several taxonomies and are curated from both low and high throughput experiments. As of 2005, BIND contained approximately 180,000 interactions. The site is updated daily with new interactions, but the official number is not provided on the website (Alfarano et al. 2005). This database provides four first neighbors and 87 second neighbors for NEDD9 (244 total interactions), all derived from *Homo sapiens*.

TABLE 16.1. Bioinformatics resources discussed in the text.

Tools and Databases	Web addresses
Protein–Protein Interactions	
The JCB Protein–Protein Interaction Website	www.imb-jena.de/jcb/ppi/jcb_ppi_databases.html
1. BIND	bond.unleashedinformatics.com
2. BioGRID	www.thebiogrid.org
3. HPRD	www.hprd.org
4. STRING	string.embl.de
5. DroID	www.droidb.org
Pathways	
Pathguide	www.pathguide.org
1. GenWay	www.genwaybio.com/index.php
2. KEGG	www.genome.jp/kegg
3. LINNEA Pathways	www.invitrogen.com/site/us/en/home/LINNEA-Online-Guides/LINNEA-Pathways.html
Conversion Tools	
Clone/Gene ID Converter	idconverter.bioinfo.cnio.es
FLIGHT	flight.licr.org
Visualization Tools	
Cytoscape	www.cytoscape.org
IM Browser	proteome.wayne.edu/PIMdb.html
Osprey	biodata.mshri.on.ca/osprey/servlet/Index
Other	
BIOCONDUCTOR	www.bioconductor.org
Entrez Gene	www.ncbi.nlm.nih.gov/sites/entrez
GEO	www.ncbi.nlm.nih.gov/geo
Oncomine	www.oncomine.org

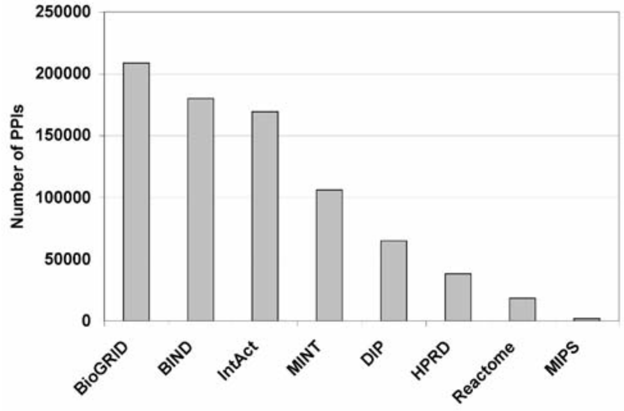


FIGURE 16.5. Numbers of interactions reported in protein interaction databases. Values represent statistics reported on each website (as of May 2008), or in recent database-linked publications.

BioGRID, the General Repository for Interaction Datasets, is a collation of curated protein and genetic interactions from six different species, including *Saccharomyces cerevisiae, Caenorhabditis elegans, Drosophila melanogaster*, and *Homo sapiens*. It contains approximately 141,500 non-redundant interactions (as of June 1, 2008 [Stark et al. 2006]). This database is updated monthly with newly identified interactions, derived from both high-throughput studies and conventional focused studies. Using only *Homo sapien* interactions to identify first and second neighbors for NEDD9, we found 27 first neighbors and 402 second neighbors (429 nodes total), with 75 edges for the first neighbors and 1806 edges in sum.

The Human Protein Reference Database (HPRD) contains PPIs as well as posttranslational modification data. HPRD has the most comprehensive collection of human PPIs of any database. This database also has other features, including isoform and functional information, sub-cellular localization, and disease association. All of the data provided in HPRD has been curated manually by reading publications reporting in vivo and in vitro experiments. As of May 2008, HPRD contains 38,167 interactions (Peri et al. 2003; Mishra et al. 2006). This database identified 29 first neighbors and 876 second neighbors for NEDD9, with a total of 5779 interactions.

All data concerning NEDD9 derived from these three databases was imported into Cytoscape by downloading the complete binary interaction files from BIND, BioGRID, and HPRD (Fig. 16.6). These imported files are then used to create networks within Cytoscape. Downloading the binary files into a single resource is more practical than using the web-browser interface for each isolated database because we can customize one aggregated file to our needs, and then generate many focused networks containing tailored attributes. The networks that we generate from the binary interaction files allow us to also retrieve the interactions between, for example, the first neighbors, whereas the web-browser interface allows us to retrieve only the interactions *between* the protein of interest and its first neighbors, but not *among* the first neighbors.

Some available databases are amalgamations of other existing PPI databases. For example, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) contains approximately 1.5 million proteins from 373 species (as of May 1, 2008) and is freely available (von Mering et al. 2007). STRING gathers interactions from sources including BioGRID, DIP, MINT, KEGG, IntAct, Flybase, and others (see von Mering et al. 2007 for a complete list). For a given species, STRING also includes its own algorithm-based predictions of interactions based on orthologous interactions observed in model organisms. When querying for interactions of a protein(s) in STRING, the web interface allows for the selection of method(s) by which the interactions of your network will be generated (i.e., experimental, text mining, orthologous predictions, etc.). This “metasearch,” as compared to the direct search of the unique databases, is invariably easier and faster, and may pick

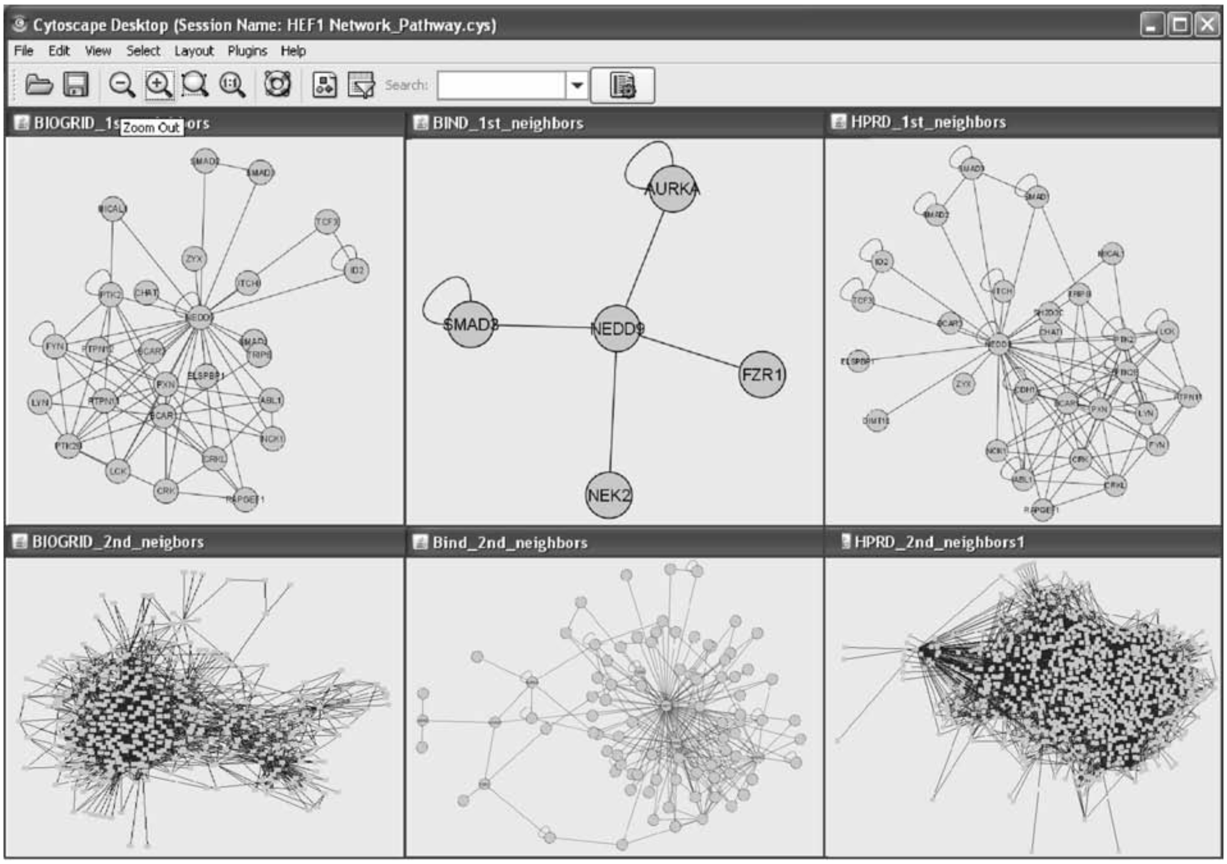
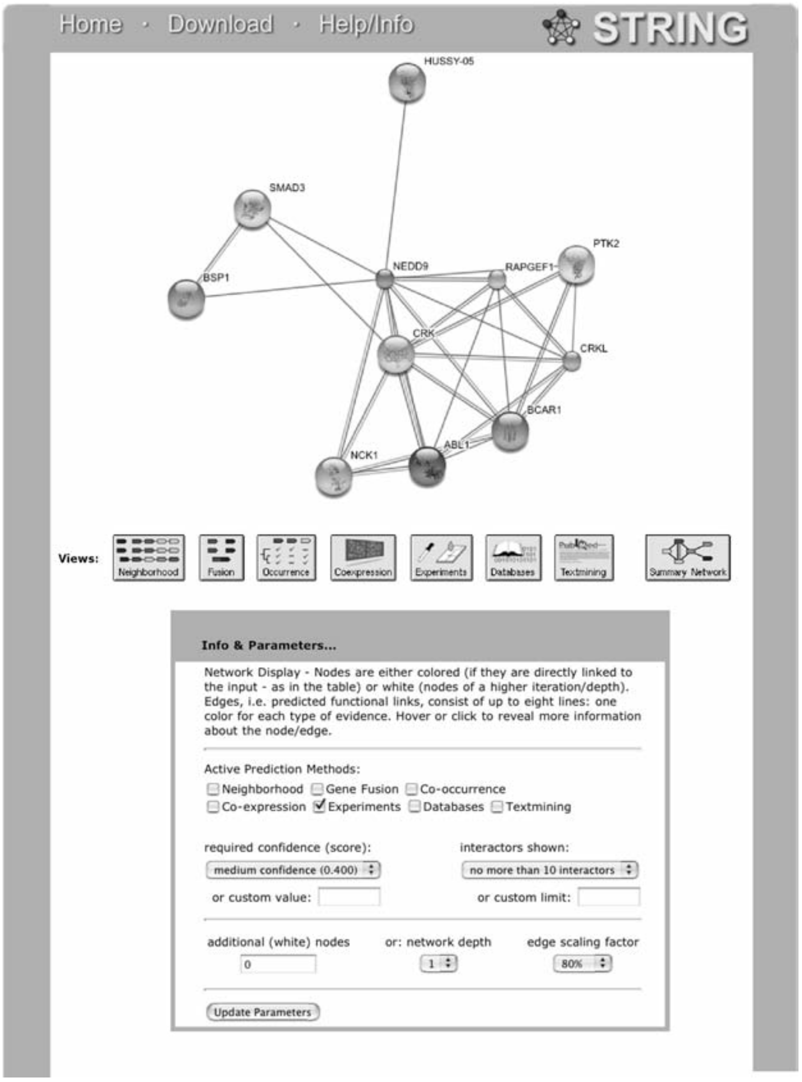


FIGURE 16.6. Assembly of different PPI datasets in Cytoscape. First and second neighbors from HPRD, BIND, and BIOGRID are displayed. (Color images displayed at http://www.fccc.edu/research/labs/golemis/Golemis_2008/CSHL_chapter.html.)

up additional interactions. However, it can also miss some interactions. It is up to the individual researcher, in defining a working network, to decide how comprehensively to search.

We chose to use a strict criterion to gather the first and second neighbors of NEDD9 from STRING, only allowing interactions that have been experimentally verified in low throughput experiments (Fig. 16.7). We found 30 first neighbors, with 92 interactions among the 31 proteins. When converting these 30 proteins into Entrez IDs, we lose five of them in the automated process, making it necessary to use manual conversion to recover them. In our STRING network first neighbor analysis we imported a table with 26 nodes and 86 edges. We used the same criteria but increased network depth parameter to 2 when performing the analysis for the second neighbors. After the ID conversion, we had a combined total of 175 nodes and 720 edges for the first and second neighbors of NEDD9 in STRING. In this case, we did not download a binary interactions file from STRING because we could retrieve the interactions between the first and second neighbors using the web-browser interface.

To complete the assembly of direct PPI information for our network, we merged the first neighbor analyses from the four databases to generate our First Neighbor Core, 33 proteins and 133 interactions among them (Fig. 16.8). It is possible to click directly on the line connecting two nodes, and directly recover information describing the source and data quality supporting the assignment of any given interactions. The merged second neighbor set developed from this core included 922 proteins and 5375 interactions. At this point, options are to include this entire dataset in the network, or to use it selectively in designing a working network. Either approach can be taken. In our work, we typically use the second neighbor set selectively to enrich biological



Info & Parameters...

Network Display - Nodes are either colored (if they are directly linked to the input - as in the table) or white (nodes of a higher iteration/depth). Edges, i.e. predicted functional links, consist of up to eight lines: one color for each type of evidence. Hover or click to reveal more information about the node/edge.

Active Prediction Methods:

□ Neighborhood □ Gene Fusion □ Co-occurrence
□ Co-expression ☑ Experiments □ Databases □ Textmining

required confidence (score): medium confidence (0.400)
or custom value:

interactors shown: no more than 10 interactors
or custom limit:

additional (white) nodes: 0
or: network depth: 1
edge scaling factor: 80%

Update Parameters

FIGURE 16.7. Screen shot, STRING search for NEDD9 interactors. The figure illustrates a simple network that was generated in a search in which only the parameter "experiments," reflecting protein–protein interaction information is considered. Other screening options ("neighborhood," "coexpression," etc.) are shown. Values such as confidence interval allow weighting of recovered results to change certainty of displayed information. (Color images displayed at http://www.fccc.edu/research/labs/golemis/Golemis_2008/CSHL_chapter.html.)

processes of functional interest for NEDD9 studies based on convergence with orthogonal datasets, as we discuss later. Finally, in the analysis described here, we specifically searched for information regarding human NEDD9. Many of the same databases contain additional information from other mammals and can often supplement human datasets.

PROTEIN COMPLEXES

The composition of the protein complexes isolated using immunoprecipitation or TAP techniques also provides clues regarding potential interaction partners. Among the PPI databases, BIND and IntAct both provide information on protein complexes. BIND has a separate subdivision of the database, whereas IntAct has both binary and intercomplex interactions merged together.

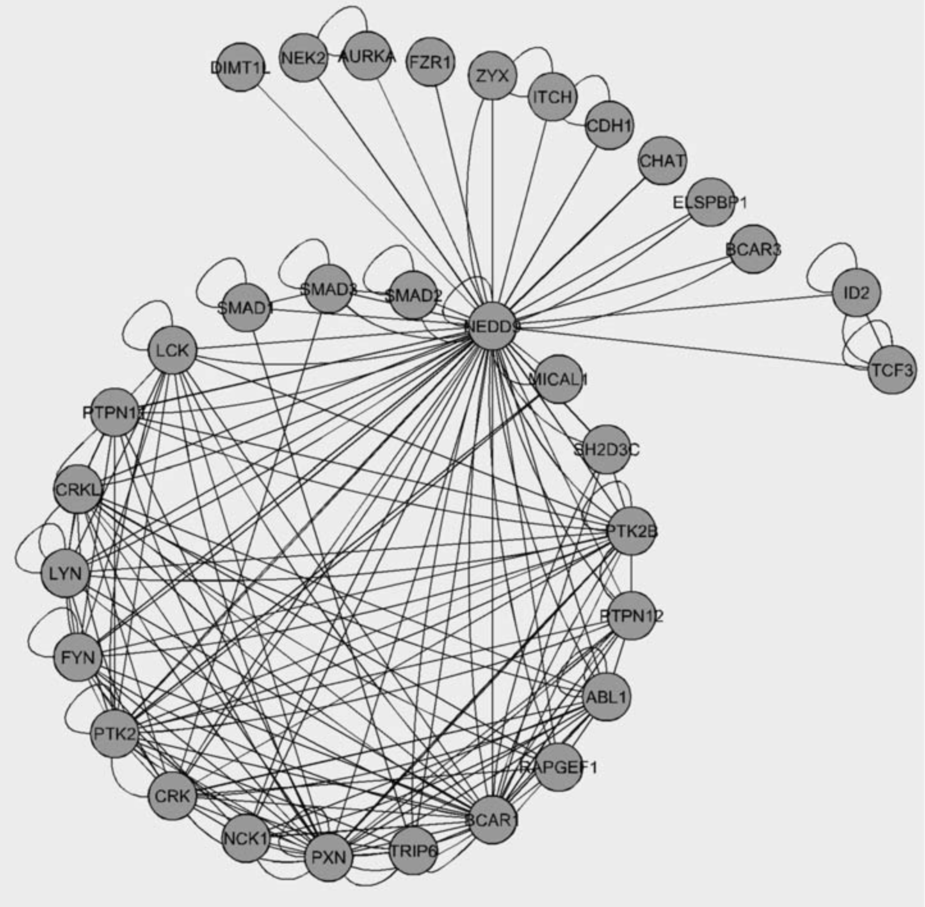


FIGURE 16.8. First neighbor core. Highly validated nodes found to interact with NEDD9 in multiple PPI databases are shown. Interactions among proteins within the group are also indicated; note some nodes are characterized by multiple cross-linkages, reflecting participation by group members in a signaling pathway (see Fig. 16.13), whereas in the absence of additional information groups, others are not. (Color images displayed at http://www.fccc.edu/research/labs/golemis/Golemis_2008/CSHL_chapter.html.)

Therefore, conducting a search for confirmed binary PPIs is more challenging in this database if the intent is to separate these two categories. Unlike the binary interactions reported in the previous examples, however, these data do not provide information as to whether the interactions are direct or indirect. Hence, depending on the goal of the experiments, it may not be a good idea to look for second neighbors of proteins retrieved as components of a complex. In addition, PPI databases sometimes miss protein complexes described in the research literature, perhaps because of the difficulty of effectively annotating interactions involving a protein complex in the absence of agreed-upon naming conventions. Thus, these data likely represent an underestimate of what is available via PubMed searches. In searching for protein complexes involving NEDD9, we find one complex described in the literature (BIND ID:144540). Most of the components of this complex were already known from a binary interaction search, but one new interaction, APC10, was added.

"CANONICAL PATHWAYS"

It is possible to continue expanding the networks around a protein of interest by searching for third neighbors in the PPI databases. In practice, however, this search produces such a large dataset as to remove utility. Even the list of second neighbors is frequently unwieldy for generating hypotheses. As a complementary approach, it is useful to matrix PPI data with information from "canonical pathway" databases, constructed either as free community resources, or as commercial products. The rationale in this approach is that whereas there is a limited number of research articles addressing the functionality of NEDD9, some of NEDD9's well-validated first neighbors have attracted significant interest. Resources that address these neighbors can identify functional and highly-validated partners as well as upstream or downstream factors that are relevant to NEDD9, and this information might illuminate subsets of the second neighbor dataset of particular interest for specific biological processes.

One comprehensive source providing a point-of-entry to signaling pathway databases is Pathguide (Bader et al. 2006). We initially searched directly for pathways that contained NEDD9 based on our knowledge of its involvement of specific biological processes (i.e., focal adhesion signaling), or, alternatively, we directly queried for NEDD9 in the search function of a more extensive group of databases. These approaches did not identify a hit for NEDD9 in a search of over 25 different pathway databases. However, from our initial PPI analysis, we knew that NEDD9 binds and is phosphorylated by SRC in the focal adhesion pathway, and that SRC is an important and relevant regulator of cancer metastasis. We therefore collected the nodes around SRC in the canonical focal adhesion signaling pathways from three databases to identify key proteins in these processes. This approach proved to be extremely productive.

The three focal adhesion pathway sources that we used to build this network are KEGG Pathways (Kanehisa et al. 2006) (Fig. 16.9), reflecting the work of non-profit investigators, and commercial but publically accessible options such as GenWay Pathways and Linnea Pathways. These (and many other) databases do not provide a file containing all the binary interactions of

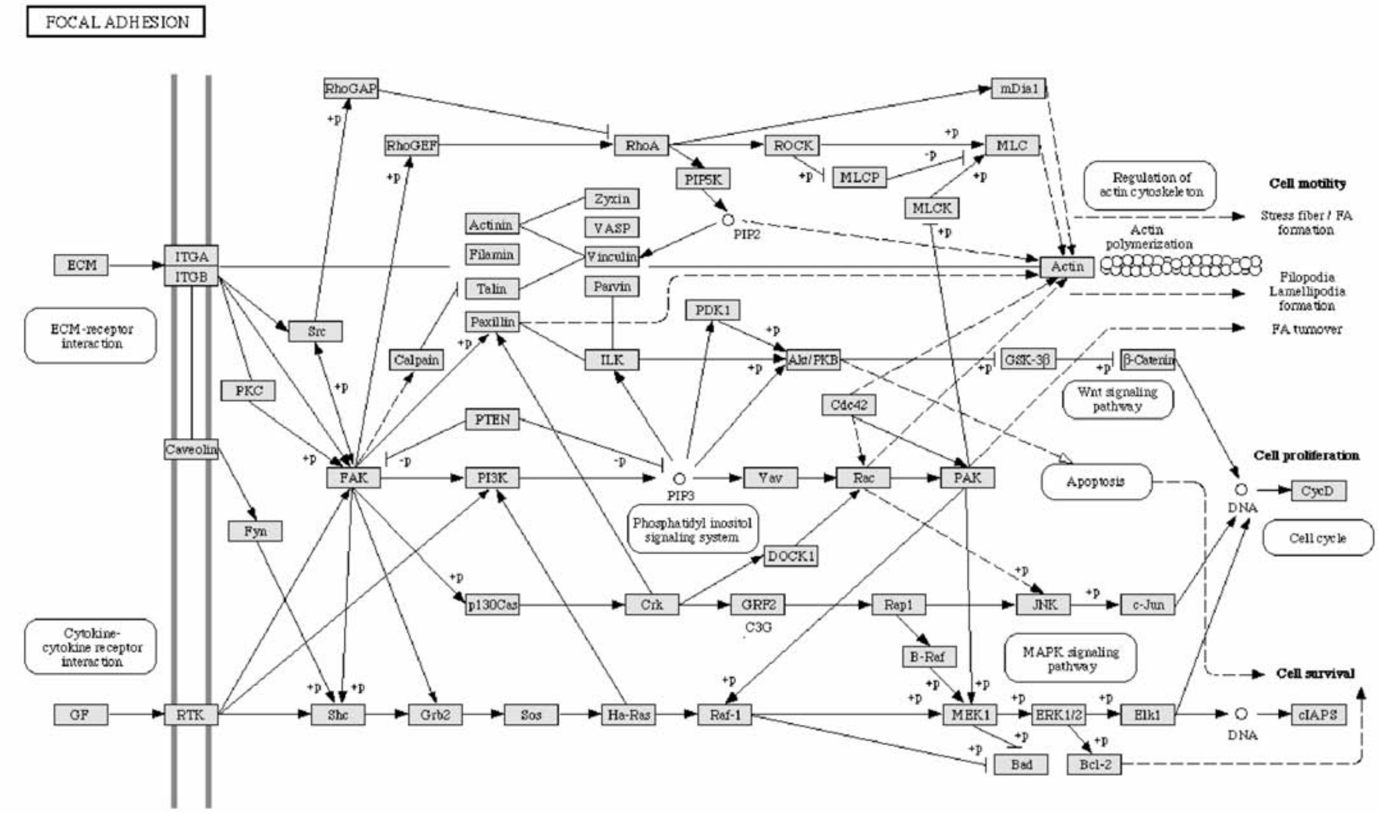


FIGURE 16.9. Focal adhesion pathway map from KEGG (Kanehisa et al. 2006). Although NEDD9 is not found in this map, several of its high confidence first neighbors (e.g. FAK, SRC family kinases, p130Cas) are present. The data is collected from functionally linked proteins.

the pathway. Rather, nodes in these pathways are initially captured as lists, and if desired, re-mining of PPI databases is exploited to fill in the interactions among key nodes. This step of creating the network can be time consuming if pathway databases of particular interest do not automatically provide a list of genes/proteins involved in the pathway (which is typical). It therefore may be necessary to generate a list manually of genes/proteins in the pathway from the image available. Once a list is obtained, the gene symbols are converted to Entrez IDs using the Clone/Gene ID Converter, discussed previously (Alibés et al. 2007). Note that the pitfalls we discussed for automated and manual name conversion apply here also. For example, only approximately 66% of the genes from the Linnea Focal Adhesion Pathway were automatically converted, with the remainder requiring manual search for Entrez ID number.

Once lists of genes and proteins from the three pathway programs have been converted to Entrez IDs, the datasets are merged as for PPIs. For NEDD9, this process identified 42 unique nodes. Only four of the nodes were represented in all three focal adhesion pathways, representing the somewhat subjective biases underlying inclusion criteria of these pathways. However, 15 nodes were found in at least two of the three pathways, suggesting a higher level of confidence in direct functional relevance. Comparing these 42 nodes with the PPI first and second neighbor datasets, we find that 23 of the nodes are encompassed within the second neighbor set, including ten validated by at least two pathway databases. This observation suggests that these 23 nodes, and their interacting partners within the first and second neighbor set (Fig. 16.10), might be a valuable set of genes to consider as a group when evaluating NEDD9 function in metastases. We added these 23 nodes to the NEDD9 "core" network of PPIs, bringing it to 57 nodes. A similar approach can readily be applied to other NEDD9 first neighbors associated with biological processes of particular interest.

INSIGHT FROM MODEL ORGANISMS

Model organisms such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster* have long been exploited because of their powerful genetics, which reveals functional relationships among proteins. Further, model organisms were the first to be exploited in high throughput two-hybrid (Uetz et al. 2000; Ito et al. 2001; Giot et al. 2003; Li et al. 2004) and mass spectrometry/complex purification studies (Gavin et al. 2002, 2006). Proportionally, a much higher percentage of proteins is functionally annotated in these organisms than in higher eukaryotes, and very powerful curated databases provide convenient sources for data retrieval (see the list of websites in Table 16.1). Because of evolutionary constraints, many important physical and functional relationships are known or likely to be conserved between lower and higher eukaryotes, and judicious compilation of the interaction data across multiple species can increase the diversity of the nodes within a focused network. The term interolog describes a protein–protein interaction that is common between species: This term was first proposed by Yu et al. (2004) and the value of interolog analysis has been described in several key studies (Bandyopadhyay et al. 2006; Sharan et al. 2005; Ulitsky and Shamir 2007).

In exploiting these resources for our network construction, we note that NEDD9 is one of a family of four proteins in mammals. This family has a single ancestral family member in

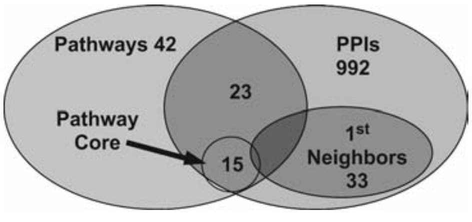


FIGURE 16.10. Pathway/PPI convergence in identification of NEDD9-relevant nodes. See text for details. (Color images displayed at http://www.fccc.edu/research/labs/golemis/Golemis_2008/CSHL_chapter.html.)

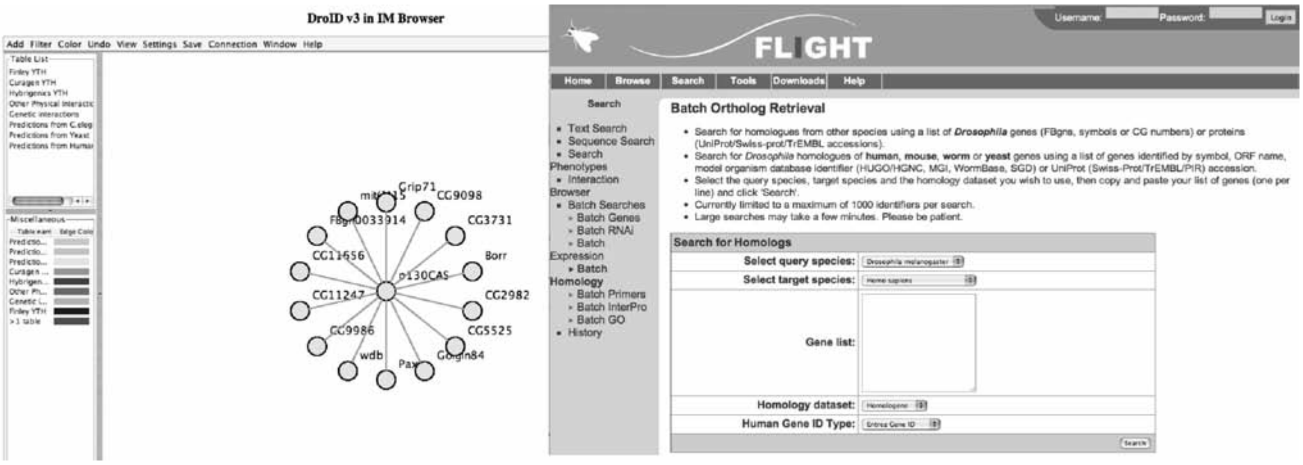
Drosophila, and no clear relatives in *Caenorhabditis* or *Saccharomyces*. We therefore first used the *Drosophila* Interactions Database (DroID) to find known interactions with the NEDD9 *Drosophila* homolog, CG1212 (Pacifico et al. 2006). We excluded the human interolog prediction criterion but allowed all other methods of generating interactions. Using this approach, we identified 14 interactions among 15 unique nodes for the first neighbor analysis. Among the 15 unique nodes, five have human homologs. Expansion to DroID second neighbors yielded 340 total nodes for the CG1212 network. We then looked for human homologs using the FLIGHT database (Sims et al. 2006), which provides a central hub and many useful tools for the compilation of high-throughput *Drosophila* data. Among the 340 nodes, 170 have human homologs, as predicted by the Homologene search tool from the FLIGHT database (Fig. 16.11).

Three of these 170 homologs intersect with the first neighbor and 15 with the second neighbor human PPI dataset; the remaining homologues are novel. This data clearly provides an additional level of confidence due to the 15 nodes identified in the second neighbor analysis, which are likely to be important for core NEDD9 gene family functions. However, as we saw, there are four parallel CG1212 related genes with non-identical function in humans, and the biology of flies is non-identical to that of humans. We therefore have a lower level of confidence that the 155 novel nodes are directly relevant to NEDD9 function. Yet, this group can be used to fill in other interaction clusters of interest as we shall see in the final two sections of the chapter (see The Assembled Network and Using Network Tools on pp. 341 and 344).

EXPRESSION DATA: PROVIDING CONTEXT

For the human NEDD9 gene, the described approaches to network construction define a large sphere of potential functional interactions. Most of these approaches offer no clues as to whether the interacting proteins are dynamically co-regulated for specific biological processes or disease states. It is well known, however, that biological networks generate coordinated changes in gene expression. Hence, an important orthogonal dataset that can be used to inform the NEDD9 network describes gene and protein expression.

Most journals now require authors to deposit microarray datasets for public access together with a minimal set of annotations (MIAME) (Brazma et al. 2001). Most of the published microarray datasets are publicly available in the ArrayExpress database (Brazma et al. 2003) or in the Gene Expression Omnibus (GEO) (Edgar et al. 2002; Barrett et al. 2006). In the GEO database, the expres-



Batch Ortholog Retrieval

- Search for homologues from other species using a list of ***Drosophila*** genes (FBgns, symbols or CG numbers) or proteins (UniProt/Swiss-prot/TrEMBL accessions).
- Search for *Drosophila* homologues of **human**, **mouse**, **worm** or **yeast** genes using a list of genes identified by symbol, ORF name, model organism database identifier (HUGO/HGNC, MGI, WormBase, SGD) or UniProt (Swiss-Prot/TrEMBL/PIR) accession.
- Select the query species, target species and the homology dataset you wish to use, then copy and paste your list of genes (one per line) and click 'Search'.
- Currently limited to a maximum of 1000 identifiers per search.
- Large searches may take a few minutes. Please be patient.

Search for Homologs

Select query species:	Drosophila melanogaster
Select target species:	Homo sapiens
Gene list:	
Homology dataset:	Homologene
Human Gene ID Type:	Entrez Gene ID

FIGURE 16.11. The DroID tool in FLIGHT database. See text for details.

sion levels of genes in an experiment can be examined using the GEO Profiles tool (Barrett et al. 2006), which searches for a gene of interest, and returns microarray datasets in which its mRNA is differentially expressed. These results can then be used to identify mRNAs having either similar or opposite expression profiles (Barrett and Edgar 2006). In turn, these gene products can be compared with the PPI proteins identified from other datasets. Because these searches are performed across all datasets, the results can suggest interesting model systems in which to functionally analyze your protein.

In addition to the ArrayExpress and GEO international repositories, cancer researchers use an oncology-specific database, Oncomine (Rhodes et al. 2007), which is free for non-profit researchers and provides easy searches to retrieve datasets in which expression of a specific gene is differentially regulated. A NEDD9 search in Oncomine returns all cancer datasets in which NEDD9 is differentially regulated at a defined *p* value threshold. The summary displays the differential activity map, with red and blue showing overall up-regulation or down-regulation, respectively, of NEDD9 in various tissue types (Fig. 16.12a). Each number represents the number of studies for that specific tumor type and specific comparison type that show differential regulation of the target gene. Choosing the number using the mouse leads to the dataset.

As an example, we chose the Head and Neck study showing mild up-regulation of NEDD9 in cancer versus normal tissues. We can then represent expression by producing either a box plot or a cancer profile outlier plot (see Fig. 16.12b), which clearly shows that, in this study, NEDD9 is likely to be more highly expressed in tumors than in normal tissues.

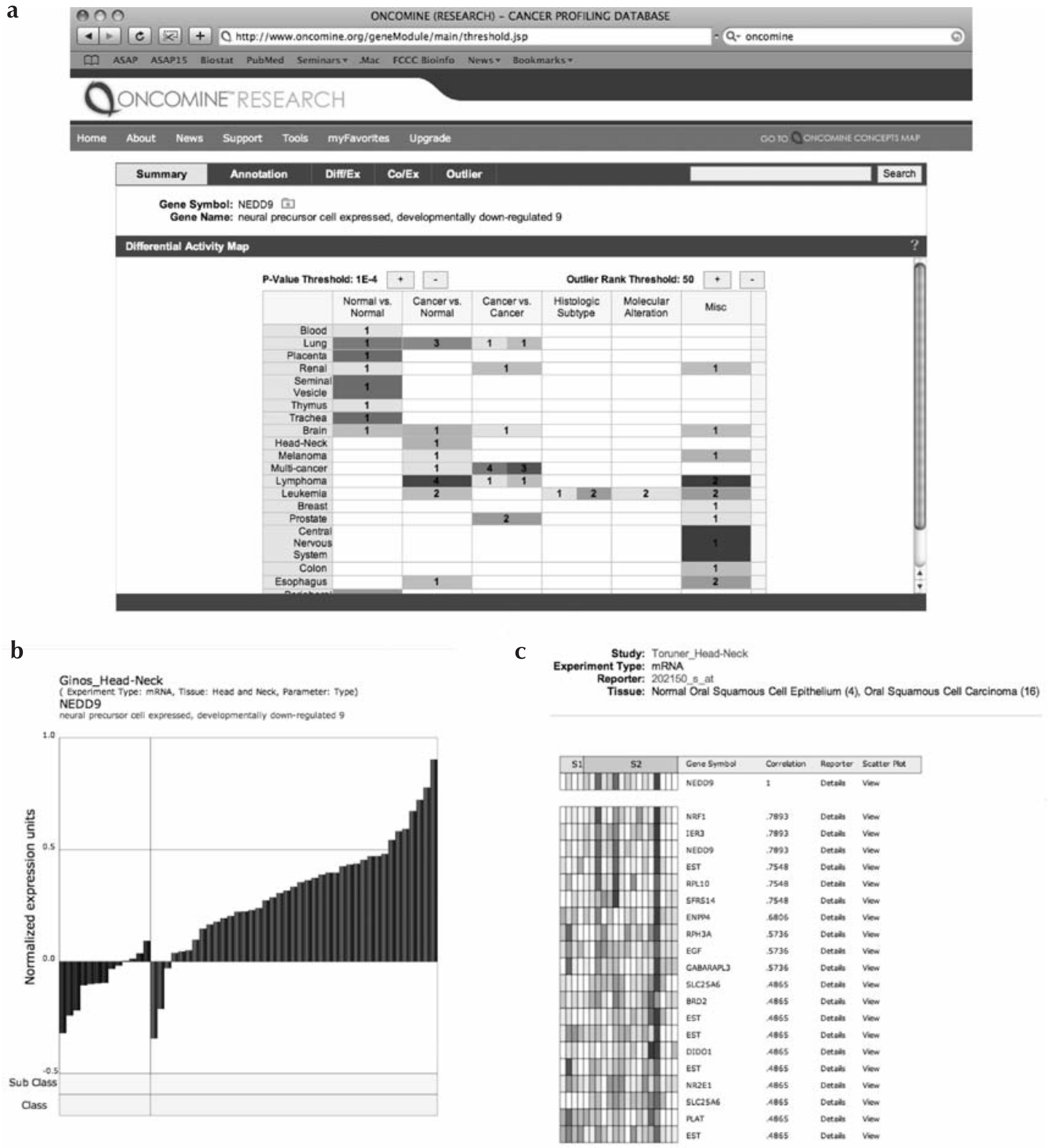
Genes that behave similarly to the target gene within a study can also be identified by using the Co/Ex option in Oncomine: Genes whose expression profiles are highly correlated with that of the target gene are reported. This option is not available for all studies in Oncomine, including that shown in Figure 16.12b; however, data for a second Head and Neck cancer study is available (see Fig. 16.12c). In this example, the expression profile of NEDD9 was identified as similar to that observed for other gene products, including NRF1, IER3, RPL10, and others. These gene IDs provide another orthogonal dataset that can be overlaid with the other predictive methods described here.

As a cautionary note, it is important to recognize the diversity in the quality of microarray data. Many datasets were published when microarray technology first emerged, but obviously both the core technology and analytic methodology have improved significantly in recent years. Re-analyzing older raw data using current methods is a good precaution if you are considering including this data in a network. For statistical and informatics researchers, the R/Bioconductor package provides advanced tools for microarray data analysis, which can be automated to quickly retrieve, reannotate, and reanalyze data from GEO (Gentleman et al. 2004).

THE ASSEMBLED NETWORK: USING THE NEDD9 LITERATURE TO ESTABLISH NETWORK UTILITY

One reason we selected NEDD9 (also known as HEF1 and CAS-L) as an example for this analysis is that our research group has been studying this protein for 15 years and we are extremely familiar with its biology (O'Neill et al. 2007; Singh et al. 2007). This accumulated knowledge allows us to examine the data generated through the resources described above and to assess how effectively the main themes in NEDD9 have functionally been captured in the *in silico*-generated network.

Figure 16.13 shows one representation of an assembled NEDD9 network. In this representation, we have included two additional sets of information with the data assembled thus far. In one case, we have used text-mining (Ananiadou et al. 2006), an option easily selected in STRING, to find gene names significantly linked to NEDD9 in the scientific literature. Given the current state of the art, this data is of lower confidence than the other data gathered from programs we have described. In Figure 16.13, text-mined "interactions" with NEDD9 are indicated with dashed lines, and nodes are shown in paler colors. We have also included data from Aceview (Thierry-Mieg and Thierry-Mieg 2006), a program which compiles information about genes based on co-expression,



Gene Symbol: NEDD9
Gene Name: neural precursor cell expressed, developmentally down-regulated 9

P-Value Threshold: 1E-4
Outlier Rank Threshold: 50

	Normal vs. Normal	Cancer vs. Normal	Cancer vs. Cancer	Histologic Subtype	Molecular Alteration	Misc
Blood	1					
Lung	1	3	1 1			
Placenta	1					
Renal	1		1			1
Seminal Vesicle	1					
Thymus	1					
Trachea	1					
Brain	1	1	1			1
Head-Neck		1				
Melanoma		1				1
Multi-cancer		1	4 3			
Lymphoma		4	1 1			2
Leukemia		2		1 2	2	2
Breast						1
Prostate			2			1
Central Nervous System						1
Colon						1
Esophagus		1				2

b
Ginos_Head-Neck
(Experiment Type: mRNA, Tissue: Head and Neck, Parameter: Type)
NEDD9
neural precursor cell expressed, developmentally down-regulated 9
Normalized expression units
1.0
0.5
0.0
-0.5
Sub Class
Class
c
S1
S2

Study: Toruner_Head-Neck
Experiment Type: mRNA
Reporter: 202150_s_at
Tissue: Normal Oral Squamous Cell Epithelium (4), Oral Squamous Cell Carcinoma (16)

Gene Symbol	Correlation	Reporter	Scatter Plot
NEDD9	1	Details	View
NRF1	.7893	Details	View
IER3	.7893	Details	View
NEDD9	.7893	Details	View
EST	.7548	Details	View
RPL10	.7548	Details	View
SFRS14	.7548	Details	View
ENPP4	.6806	Details	View
RPH3A	.5736	Details	View
EGF	.5736	Details	View
GABARAPL3	.5736	Details	View
SLC25A6	.4865	Details	View
BRD2	.4865	Details	View
EST	.4865	Details	View
EST	.4865	Details	View
DIDO1	.4865	Details	View
EST	.4865	Details	View
NR2E1	.4865	Details	View
SLC25A6	.4865	Details	View
PLAT	.4865	Details	View
EST	.4865	Details	View

FIGURE 16.12. Typical results from Oncomine. These screenshots from the Oncomine resource demonstrate (*a*) the results of a search for NEDD9 in the database, (*b*) a Cancer Outlier Profile Analysis (COPA [MacDonald and Ghosh 2006]) projection for NEDD9 in the Oncomine database, using the Ginos Head and Neck tumor study as source, and (*c*) use of the profiling function to discover genes with similar expression profiles to NEDD9 expression in the Toruner Head and Neck tumor study. (Color images displayed at http://www.fccc.edu/research/labs/golemis/Golemis_2008/CSHL_chapter.html.)

gene ontology (GO) classification, common domains, protein interactions, and other parameters. One of its unique features allows the program to make predictions about likely functional interactions based on convergent datasets between a query protein and other proteins. We have included cases (shown by nodes with white centers in the figure) for which interactions predicted by Aceview (using all search criteria *except* protein–protein interactions) overlap with genes in the NEDD9 "second neighbor" sphere of interactors.

Well-established themes in the study of NEDD9 have been (1) interaction with integrin-dependent signaling cascades that control migration, invasion, and cell survival; and (2) integra-

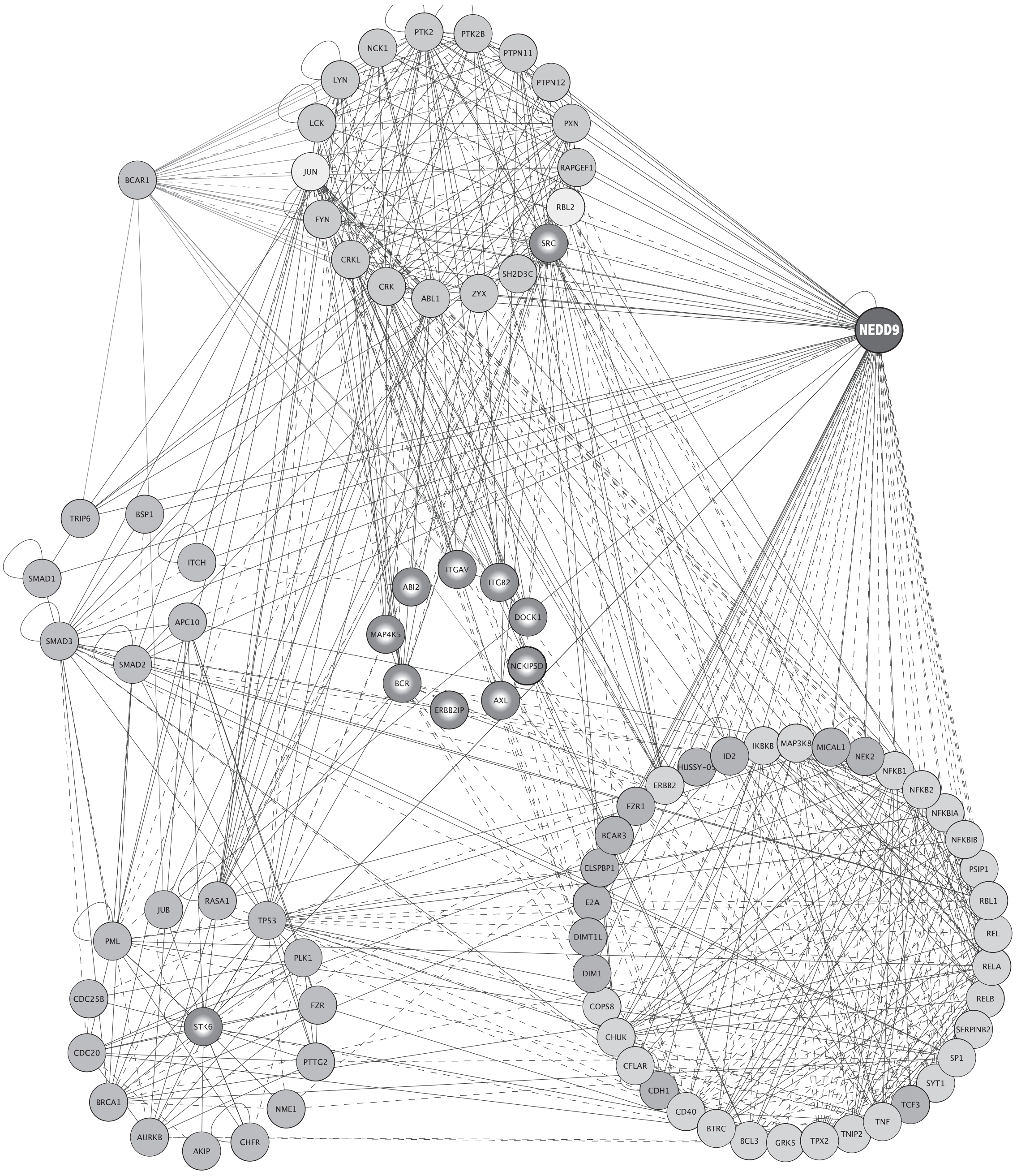


FIGURE 16.13. Composite NEDD9 network. As described in the text, this network contains PPIs, networks, Aceview predictions, and text-mining. The "live" Cytoscape resource and a color version of the figure can be viewed at the Golemis lab website, http://www.fccc.edu/research/labs/golemis/Golemis_2008/CSHL_chapter.html.

tion with multiple components of the TGF-β signaling machinery, relevant to cell differentiation control. Using the dynamic features of Cytoscape, we have moved many of the proteins implicated in these NEDD9 activities into two separate clusters. The circles at the top center represent proteins involved in integrin-dependent signaling; those at the center left represent a cluster of TGF-β signaling effectors, including APC10 (identified from protein complexes). As well as demonstrating the direct interactions of each of these proteins with NEDD9, this clustering effort easily identifies associations among the proteins within the functional group. It is also possible to readily display interactions that are known to be common between NEDD9 and its paralog, BCAR1/p130Cas (*upper left*, single node). Clearly, many of the interactions with the integrin-dependent signaling set are conserved between NEDD9 and BCAR1, whereas interactions with the TGF-β signaling machinery are thus far apparently specific to NEDD9.

Interestingly, most of the protein interaction- and expert knowledge-based resources miss an important and well-documented NEDD9 interaction relevant to integrin signaling, that between NEDD9 and SRC. Similarly, the interaction between NEDD9 and Aurora-A kinase/STK6 (first reported in 2005) connecting NEDD9 to pathways controlling mitosis and ciliary dynamics (Pugacheva and Golemis 2005; Pugacheva et al. 2007) is not detected by STRING due to a gap in this database. However, Aceview clearly predicts the SRC and Aurora-A interaction (nodes with white centers), as well as a number of other potential interactions involving proteins such as ABI2, ITGAV, and others that show multiple interactions with NEDD9 "first neighbors."

Selecting Aurora-A as an example of particular interest, we then mined Aurora-A "first neighbors," and compared this list to the set of genes already known to be included within the NEDD9 first neighbor/expert system network, or to the set of proteins predicted by text-mining to be NEDD9 partners. A large number of these Aurora-A first neighbors were found to interact with one or multiple proteins that have also been connected to NEDD9. These findings suggest possible hypotheses for how NEDD9 functions in different processes. Finally (*lower right*), additional NEDD9 interactions affecting a diverse set of cellular processes are predicted by various other means. The density of interactions among proteins in this group suggests functional clusters (for example, among a group of text-mined proteins associated with inflammatory response). However, with network updating, proteins we have somewhat arbitrarily clustered within this "other" group may emerge as being involved in a new functional process. Similarly, specific scrutiny of one partner of interest (chosen arbitrarily, e.g., MICAL) may identify new bridges connecting these "other" proteins more firmly to one of the well-established NEDD9 functions. The network therefore provides a dynamic resource that helps us to organize our thinking and suggest new research directions.

USING NETWORK TOOLS TO ANALYZE CUSTOM, EXPERIMENTALLY DERIVED PROTEIN INTERACTION SETS

As of 2008, many molecular biologists and biochemists are likely not fully aware of the extent of high quality data that is freely available to support the studies of most known proteins. Highly specialized skills are not necessary: The authors learned to use the applications described here during 1–2 months of "playing" with online programs and following instructions accessible on free websites. Starting from the NEDD9 seed, we were able to generate a rich resource for its associated protein interactions. By benchmarking to the available literature on this protein, we see that these data mining efforts readily captured all direct interactions previously noted in the literature in a highly manipulable graphical display tool. The power of the approach emerged with the move toward the NEDD9 second neighbors. Because of time constraints, it is clearly not feasible to use literature-based search in adequate detail to perform direct neighbor analysis on all of NEDD9's first neighbors. However, by performing the simple steps to identify the NEDD9 second neighbors and cross-referencing this data to other resources, we were able to identify a large set of candidate interacting proteins. These candidates might very reasonably be predicted either to bind directly to NEDD9, or to be closely connected to its function, thereby identifying high-density interactions among the proteins in this group. A review of related publications in PubMed suggests that many of these candidates (e.g., ABI2, AXL, p53, or PML) have never been directly studied in the context of NEDD9 biology. Nevertheless, when we consider the density of direct physical connections and, in some cases, co-expression and other available data clearly visible from the interaction modeling, it seems extremely likely that these proteins may indeed regulate or be regulated by NEDD9 in a relatively direct way. These and other hypotheses remain to be tested.

There is not as yet (in 2008) a substitute for direct wet bench experimentation to identify novel PPIs or probe pre-existing ones. The approach described here does not work well with proteins that have attracted no prior research attention. For example, we chose two unstudied genes (LOC653352 and LOC63920), and, using these to search for interactions in STRING, BioGRID, HPRD, and BIND, we retrieved no hits. However, the depth of our understanding of the cellular machinery has changed vastly, even in the past decade, and there is no reason to believe the rate of change will decelerate. For researchers performing the protein purification techniques described in the first part of this book, a parallel application of effort to become facile in the use of the informatics tools introduced here will enhance data analysis now being carried out, and poise research projects to take full advantage of the discoveries of the coming decade.

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