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Production of Crystallizable Fragments of Membrane Proteins

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Many membrane proteins feature autonomously folded extramembranous domains which, when isolated from the intact protein, perform biochemical functions relevant to biological activity. Whereas intact membrane proteins usually require detergent solubilization for purification, most extramembranous fragments are soluble in aqueous solution. If appropriately constructed, such fragments are often crystallizable and the resulting atomic structures can lead to important biological insight. In most instances, these fragments are produced in recombinant expression systems. To be crystallizable, molecular fragments should be uniform in composition and conformation and be available in abundance. Considerations for the production of crystallizable fragments of membrane proteins include the definition of fragment boundaries, the control of nonuniformities introduced by glycosylation or phosphorylation, and optimization of expression systems. These aspects are addressed here in general terms and in the case studies of applications to CD4, CD8, the insulin receptor kinase, and N-cadherin.

KEY WORDS: Proteolysis; deglycosylation; phosphorylation; protein expression; transmembrane proteins; CD4; CD8; tyrosine kinase; cadherin.

INTRODUCTION

Many important biological processes occur at cell membranes, and membrane bound proteins subserve many of these functions. It is a basic tenet of structural biology that a truly meaningful understanding of any biological process requires atomic-level structural knowledge about the relevant macromolecular components. Membrane proteins are notoriously difficult subjects for high-resolution structural studies, however. Their bipartite nature, partly exposed to aqueous phases and partly embedded in the hydrophobic belt of the lipid bilayer, generally makes them insoluble both in water and in organic solvents. Instead, detergents are used for solubilization and this can be a complicating factor, for example in crystallization.

There are various modes of organization of membrane proteins with respect to the lipid bilayer (Fig. 1). Many membrane proteins are "integrally folded" within the membrane. Many others are "single-pass" transmembrane proteins with autonomously folded

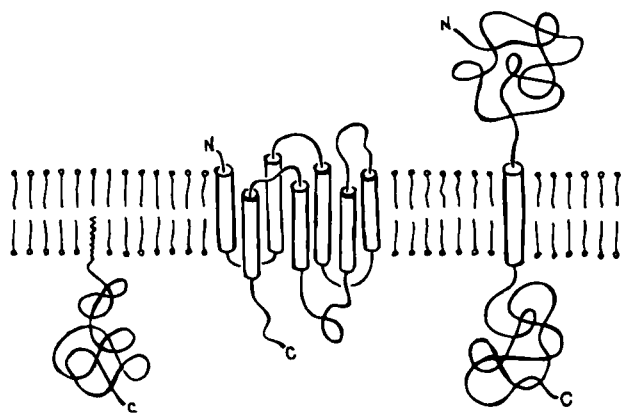

Fig. 1. Schematic illustration of three kinds of transmembrane proteins. The leftmost example is a lipid-anchored molecule. The center example represents a molecule integrally folded within the lipid bilayer, and the one on the right has a single transmembrane pass connecting domains folded in the aqueous phases on either side of the membrane.

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domains outside the membrane connected by a transmembrane segment that is predicted to be helical. Some are merely attached to the membrane by covalent lipid anchors, usually N-terminal myristyl or prenyl groups. Still others appear to insinuate themselves into or displace one leaflet of the lipid bilayer. There are many variations on these themes.

Some integrally folded membrane proteins can be studied at high resolution *in situ* by electron microscopy and diffraction as shown for bacteriorhodopsin (Henderson *et al.*, 1990) and for the light harvesting complex (Kühlbrandt *et al.*, 1994). Detergent-solubilized integral membrane proteins have also been crystallized and analyzed. Recently, the few solved examples have expanded from the photosynthetic reaction centers (Deisenhofer *et al.*, 1984; Allen *et al.*, 1987; Chang *et al.*, 1991) and porins (Weiss *et al.*, 1991; Cowan *et al.*, 1992) to include cytochrome c oxidase (Iwata *et al.*, 1995; Tsukihara *et al.*, 1995) and a bacterial light-harvesting complex (McDermott *et al.*, 1995). Other such successes are in prospect. Similarly, single-leaflet displacers can be crystallized as intact detergent-solubilized molecules, as shown for prostaglandin H synthase (Picot *et al.*, 1994).

Single-pass transmembrane proteins are especially prevalent and important at the plasma membrane of the cell surface. They include growth factor receptors such as the insulin and growth hormone receptors, cell signaling receptors such as the T-cell receptor, cell adhesion molecules such as the cadherins and integrins, and endocytic receptors such as the LDL and transferrin receptors. In each case the organization is modular. Growth factor receptors are prototypic examples. They usually have an extracellular portion which binds a specific hormone or factor and a cytoplasmic portion, often a tyrosine kinase, which activates processes in the cell when stimulated by the binding event. That these modules are separable is shown by chimeric constructions (Ullrich and Schlessinger, 1990). In many cases, the very functioning of these molecules requires fluidity within the membrane and flexibility at the membrane junctions. In other words, one does not expect that detergent-solubilized single-pass membrane proteins will be biologically meaningful entities in most cases and, since flexibility is anathema for crystallization, they will not be good candidates for structural analysis.

The modular organization of single-pass transmembrane proteins does make a reductionist, or divide-and-conquer, approach both feasible and likely to yield biologically relevant results. The extramembranous portions of such proteins follow the same folding principles as those that apply to soluble proteins, and, if cut at flexible junctions, these units can be expected to be appropriate for structural analysis. In favorable cases, extracellular fragments of this kind can be cleaved away from the membrane and the purified free fragments can be crystallized by standard methods. The earliest studies of this kind were the structural analyses of the hemagglutinin (Wilson *et al.*, 1981) and neuraminidase (Varghese *et al.*, 1983) proteins of the envelope coat from influenza virus. Another important early example came with the structure of the extracellular portion of a human Class I histocompatibility antigen (Bjorkman *et al.*, 1987). The more recent developments in expression of recombinant proteins in a variety of bacterial and eukaryotic systems has led to an explosion in the analysis of cell surface proteins. The first structures for recombinant fragments from cell surface proteins were those from the T-cell coreceptor CD4 reported independently by Ryu *et al.*, (1990) and by Wang et al. (1990). Another early structure analysis of a recombinant extramembranous fragment was an NMR study of a domain from CD2 (Driscoll *et al.*, 1991). Crystal structure analyses of fragments from CD2 (Jones *et al.*, 1992) and CD8 (Leahy *et al.*, 1992 Wang *et al.* (1990).) followed soon after. Other noteworthy structural results by this approach came with receptor–ligand complexes for the chemotactic aspartate receptor (Milburn *et al.*, 1991), for the human growth-hormone receptor (deVos *et al.*, 1992), and for the tumor necrosis factor (TNF) receptor (Banner *et al.*, 1993).

Recent successes clearly demonstrate the effectiveness of this divide-and-conquer approach to the structure analysis of single-pass transmembrane proteins. A number of potentially complicating features do need to be addressed in such studies. These include uncertainty in the appropriate definition of fragment boundaries, control of molecular uniformity with respect to post-translational modifications, and the optimization of expression systems. In this article I address each of these problems briefly and exemplify them in a series of case studies.

FRAGMENT DEFINITION

In the divide-and-conquer approach to membrane proteins the first decision to be taken concerns the limiting boundaries of the fragment for study. These can be defined theoretically with reference to sequence

information or through experiments. Since hydrophobicity profiles usually provide a clear-cut definition of the transmembrane segment, an obvious possibility is to design a construct to express an entire extramembranous portion of a transmembrane molecules. It often happens that the extramembranous portions themselves are modular with recognizable sequence repeats (Bork, 1992). In anticipation of possible flexibility at junctures between these modules, alternative constructs broken at predicted module boundaries provide another basis for definition. In some instances module boundaries are indicated by intron positions in the gene structure (Patthy, 1991) and these then become candidates to define fragment boundaries.

In the experimental approach to fragment definition, proteolysis is the principal tool. This can be done by cleavage of natural molecules from cell surfaces or alternatively by limited proteolysis of recombinant extramembranous domains. It is wise to use a battery of proteases in order to optimize the production of stable fragments, and it is useful to carry out these experiments with immobilized enzymes in order to stop reactions quickly. Yet another experimental approach is to express a variety of fragments in an activity screen to define core fragments. This can be important since small changes in termini can have profound effects on both stability and activity.

STATE UNIFORMITY

Heterogeneity, whether it be in composition or in conformation, tends to interfere with crystallization. Most membrane proteins are subject to a variety of post-translational modifications. The most important of these are extracellular glycosylation and cytoplasmic phosphorylation. When proteins are overexpressed in cultured cells, one often observes that these modifications are not uniform in the purified population of molecules. Moreover, particularly for carbohydrates, one tends to find substantial conformational disorder in crystal structures. Finally, there may be heterogeneity in polypeptide terminations in the case of proteolytic definition or conformational disorder at such a site. Crystals might grow in spite of local heterogeneity, but in essence this excludes a certain part of the surface from possible lattice contacts. If the heterogeneity can be removed, more of the surface can be available for lattice interactions.

One approach to controlling heterogeneity is to carry out expression in systems where the modifications will not occur. This can be done, for example, by expression in bacteria or in animal cells with inhibitors of modifying enzymes. Alternatively, one can attempt to remove the modified groups. For example, deglycosylation can be carried out either chemically with hydrofluoric acid or with a battery of glycosidases. It is particularly easy to remove terminal sialic acid residues, thereby substantially reducing charge heterogeneity. In most instances, the carbohydrate units have little effect on activity. The situation is quite different for phosphorylation which often has a regulatory role. Oftentimes one wishes to define particular states of phosphorylation which may be associated with overall molecular conformation and activity. It is relatively straightforward to use ion-exchange chromatography to isolate protein molecules with the same level of phosphorylation but rather more difficult to assure that this population is uniformly phosphorylated. As described in the preceding section, proteases can be used to remove flexible termini from protein fragments or, by iteration, new boundaries can be defined for the recombinant constructs.

EXPRESSION SYSTEMS

A great variety of expression systems have been devised for producing recombinant proteins (Rosenberg and Moss, 1990, and associated articles) and many of these have been adapted to the production of membrane protein fragments. Each has its own peculiar advantages and possible complications, and particular proteins might be best suited for particular expression systems. In our own experience we have concentrated on four particular distinctive expression systems. The first of these is *Escherichia coli.* Bacterial expression is relatively convenient and economical but may not be appropriate for the production of eucaryotic proteins. Recent developments in fusion proteins (Nilsson *et al.,* 1992) and in the use of molecular chaperons is, however, making bacterial expression more generally useful. Chinese hamster ovary (CHO) cells can be transfected with appropriate secretion constructs for the production of secreted mammalian proteins in a rather natural setting. This leads to glycosylation with complex carbohydrates similar to those that would obtain in the natural product. Such glycosylation events may be essential for the proper expression of these proteins. Stable CHO cell lines amplified for high level production can be established readily, but the amplification takes several weeks. Another very

popular system for the expression of eucaryotic proteins is the use of baculovirus-infected insect cells. This system has proved particularly advantageous for a number of cytoplasmic proteins, kinases in particular, but it has also been used effectively for secreted proteins. Proteins secreted from insect cells are glycosylated exclusively with high-mannose sugars, as opposed to the complex carbohydrates of mammalian cells. Such high-mannose sugars are uniquely susceptible to digestion with endoglycosidases that leave a single N-acetyl glucosamine, providing a benign residue of what might otherwise be a flexible site. Finally, the *Pichia* yeast system (White *et al.*, 1994) appears to have substantial advantages particularly for the expression of extracellular fragments. As with *E. coli* the cloning manipulations are relatively simple and economical, but since yeast are eukaryotes they may be better hosts for expression of mammalian proteins. As for insect cells, the yeast produced high mannose sugars, which can be an advantage. A particular advantage of the *Pichia* system is that these yeast can be grown on a defined medium in which methanol is the sole carbon source. This can simplify purifications.

CASE STUDIES

CD4

The initial construct used in our crystallization studies on human CD4 was a soluble form (sCD4) that was truncated at the predicted transmembrane boundary (residue 369). The construct was transfected into CHO cells and amplified. The expressed material was purified by conventional chromatographic procedures. sCD4 is glycosylated as produced in CHO cells, and there is heterogeneity in the glycosylation (Carr *et al.*, 1989). One aspect of this heterogeneity was evident by native gel electrophoresis showing a ladder of bands due to heterogeneity in sialic acid content; this could be removed by neuraminidase treatment (Kwong *et al.*, 1990). Full-length sCD4 crystallized readily, producing five types of crystals. Unfortunately, none of these diffracted very well with conditions then available (Kwong *et al.*, 1990). A variety of deglycosylation procedures were tried on sCD4 without noticeable change in crystal characteristics, which suggested to us that poor ordering in these crystals most likely reflected intrinsic flexibility in the molecule (Kwong *et al.*, 1990). It is interesting to note that with cryoprotection these crystals are now minimally suitable and the structure analysis is proceeding with MAD phasing (Hendrickson, 1992) based on the selenomethionyl protein produced in the CHO cells (Hendrickson *et al.*, 1990).

Since the extracellular part of CD4 was predicted to be composed from four domains, flexible linkages between these domains seemed possible. The analysis of proteolytic cleavage products showed special sensitivity at the predicted boundaries between domains D2 and D3 (Ibegbu *et al.*, 1989; Healey *et al.*, 1990; Ryu *et al.*, 1990). Ultimately, the expression of a recombinant construct encoding the D1D2 fragment (Arthos *et al.*, 1989) did prove to be well behaved and this led to the crystal structure reported by Ryu *et al.* (1990). A similar construct was used in the independent analysis of the D1D2 fragment by Wang *et al.* (1990). It may be important that the D1D2 fragment is not glycosylated. Analogous studies on rat D1D2 were unsuccessful although a rat D3D4 construct did lead to a successful structure analysis (Brady *et al.*, 1993).

CD8

Human CD8 was known from its sequence to have an N-terminal immunoglobulin domain followed by a 48-residue linker, a transmembrane segment, and a short cytoplasmic domain. Cysteine residues at positions 143 and 160 lead to interchain disulfide bonds in the CD8 dimer. In our attempt to find a crystallizable fragment, three recombinant constructs were expressed: one stopping at position 114 immediately after the immunoglobulin-like domain, one stopping at 146 after the first disulfide bridge, and another stopping at 162 just at the membrane interface. These constructs were introduced into CHO cells and amplified. We found no detectable secreted protein for the short, immunoglobulin fragment, and the full-length protein behaved as a monomer on nonreducing SDS gels, suggesting that an intrachain disulfide bridge had been formed. Fortunately, the intermediate 1–146 construct directed the synthesis of a secreted disulfide-linked homodimer of CD8. Although expression levels were not as high as had been achieved with CD4, sufficient protein was produced to be purified for crystallization trial.

Efforts to crystallize sCD8, as expressed, failed. We surmised that flexibility in the 32 residues C-terminal from the immunoglobulin-like domain and heterogeneity due to glycosylation might have been responsible for preventing crystallization. There are

several sites of O-linked glycosylation on the CD8 extension, and native gel electrophoresis suggested that at least four differently charged species had been produced. Neuraminidase treatment reduced this heterogeneity but the desialated material also failed to crystallize. Subsequent treatment with O-glycosidase produced a material that crystallized readily, but into extremely thin needles (< 10 μm). Next, sCD8 was incubated with a series of proteases in the hope of removing flexible termini. *Staphylococcal* V8 protease removed five residues from the C-terminus of deglycosylated sCD8, and crystals from this material grew to dimensions as large as 50 μm in cross section. This proved adequate for the structure determination (Leahy *et al.*, 1992).

Insulin Receptor Kinase

The insulin receptor is a classic growth factor receptor with an extracellular binding domain and a cytoplasmic protein tyrosine kinase domain. Based on the deduced primary sequence, a soluble 48-kD derivative (residues 959–1355) was constructed and produced in baculovirus-infected Sf9 insect cells (Wei *et al.*, 1995). The 48-kD fragment was catalytically active but attempts to crystallize this soluble derivative were unsuccessful. After observing that an initial preparation of the 48-kD fragment degraded to a shortened form with time, we carried out a series of proteolysis experiments in hopes of defining a core fragment. Digestion with a number of proteolytic enzymes led to stable active kinase fragments. The smallest of these was a 35-kD elastase fragment which was found to have residue 982 as its N-terminus. Sequencing of its C-terminus indicated that the site of cleavage was between residues 1286 and 1291 and the analysis of a 38-kD tryptic product suggested that the cleavage had occurred immediately after Lys 1283.

In an attempt to refine boundaries of the core kinase domain, a series of four N-terminal truncations and two C-terminal truncations was made and inserted into COS cell expression plasmids for transient assays of activity. Based on the results of this study, a minimal fragment which still retained full activity was constructed with deletions from both ends, to generate a 306-residue fragment (residues 978–1283). This construct was then introduced into baculovirus for infection of Sf9 cells and the ultimate expression of a crystallizable kinase domain (Wei *et al.*, 1995). The three-dimensional structure followed in short order (Hubbard *et al.*, 1994). Interestingly, although the insulin receptor kinase is a substrate for its own activity, the material produced by the insect cells proved to be completely unphosphorylated. Addition of Mg-ATP to the purified kinase domain led to autophosphorylation in a somewhat heterogeneous mixture. After stopping the reaction with a magnesium chelator, the individual states of phosphorylation could be separated by ion exchange chromatography.

Cadherin

Cadherins are cell adhesion proteins that feature a series of homologous extracellular domains. A variety of experiments had suggested that the adhesive characteristics were due to the N-terminal domain, and we set out to produce a crystallizable fragment of N-cadherin with such adhesive characteristics. The first domain of mouse N-cadherin has no cysteine residues, making it seem possible to produce this protein in bacteria. The D1 domain of N-cadherin was produced as a glutathione S-transferase fusion protein in *E. coli* (Shapiro *et al.*, 1995). Stop codons were introduced at putative domain boundaries based on an interpretation of the aligned sequences. The fusion protein was isolated by affinity chromatography on glutathione-agarose, and the desired D1 domain was isolated by cleavage with thrombin. Subsequent purification by ion exchange chromatography led to a readily crystallizable fragment. The crystal structure of this domain as reported by Shapiro *et al.* (1995) provides an interesting model of the structural basis for cell adhesion by cadherins.

PROSPECTS

It is clear that the combination of recombinant DNA technology and x-ray crystallography is having a dramatic impact in biological understanding. This is certainly the case for receptors and cell adhesion molecules which are single-pass transmembrane proteins of the kind addressed in this article. As expression systems and biochemical preparation and characterization methods improve, even greater success can be expected. There is considerable room for improvement in methods for removing heterogeneity. Prominent among future challenges will be the production of multi-chain complexes, and success is already being realized in this area (Kozono *et al.*, 1994). Clearly, a

major challenge for the future concerns the routine expression of integrally folded membrane proteins at the production levels needed for structural studies.

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