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MINIREVIEW

Alkaline Phosphatase Fusions: Sensors of Subcellular Location

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INTRODUCTION

The use of gene fusions has been one of the major components of the revolution in molecular biology and biotechnology (17, 41). Reporter genes, when fused to genes of interest, can be used to study such diverse problems as biological regulatory mechanisms, protein localization, and cellular differentiation. Gene fusions encoding hybrid proteins can also be used to clone DNA sequences which code for antigenic determinants of important proteins and to make antibodies to identify the protein products of newly isolated genes.

The range of problems which can be approached by using gene fusions has recently been expanded with the development of a new class of reporter genes. These novel approaches are based on the finding that the enzymatic or phenotypic activities of certain bacterial enzymes depend on their subcellular locations (5, 12, 26). The gene most widely used in these studies is *phoA*, coding for the *Escherichia coli* periplasmic alkaline phosphatase. The important property of alkaline phosphatase is that it is enzymatically active only when it is exported across the cytoplasmic membrane into the periplasmic space (31, 32). If certain mutations or gene fusions are used to cause alkaline phosphatase to be retained in the cytoplasm, it is enzymatically inactive (21, 26, 32). In contrast, efficiently exported alkaline phosphatase-containing hybrid proteins have enzymatic activities comparable to that of wild-type enzyme. The inactivity of cytoplasmic alkaline phosphatase appears to be due, at least in part, to the absence of essential intrachain disulfide bonds (A. Derman and J. Beckwith, unpublished results).

Because of this property of alkaline phosphatase, it can be considered a sensor for protein export signals. That is, in gene fusion systems in which various amino-terminal sequences replace the signal sequence of alkaline phosphatase, only those replacement sequences that contain protein export signals will promote transfer of the enzyme across the cytoplasmic membrane, resulting in alkaline phosphatase activity. Export signals which will promote alkaline phosphatase export include cleavable signal sequences (26), signals for export beyond the cell envelope (L. Gilson and R. Kolter, personal communication), and appropriately oriented transmembrane segments of cytoplasmic membrane proteins (27).

In addition to its novel properties as a sensor protein, alkaline phosphatase can also serve as a useful reporter gene for studies on regulation and for purification of hybrid proteins. There exist a sensitive enzymatic assay for the enzyme and a variety of indicator and selective media for doing genetic screenings and selections (40). Furthermore, an alkaline phosphatase substrate affinity column can be used in the purification of hybrid proteins (B. Traxler and J. Beckwith, unpublished results).

Two types of alkaline phosphatase fusion systems have been developed. First, there are plasmid vectors containing restriction sites located in the DNA of the *phoA* gene corresponding to the early part of the mature protein (19, 21, 24). With the appropriate restriction fragments, one can attach the amino termini of other proteins to alkaline phosphatase. Second, a transposon derivative of Tn*5* is used which contains a *phoA* gene missing its promoter, its translation initiation site, and the DNA corresponding to the signal sequence and the first five amino acids of the protein (26). When this transposon, Tn*phoA*, inserts into a gene in the correct orientation and reading frame, gene fusions coding for hybrid proteins are generated. The Tn*phoA* approach can be applied to a variety of gram-negative bacteria by using specially designed broad-host-range plasmid vectors carrying the transposon (45).

IDENTIFYING GENES FOR CELL ENVELOPE AND EXTRACELLULARLY SECRETED PROTEINS

Plasmid-encoded genes. The Tn*phoA* system has been used to identify plasmid-carried genes that code for proteins containing export signals. With a given plasmid, one can obtain a collection of insertions of Tn*phoA* which generate hybrid proteins with alkaline phosphatase activity. They are detected as blue colonies on media containing the alkaline phosphatase indicator dye, XP (5-bromo-3-chloro-indolyl phosphate). The active fusions define regions of genes on the plasmid which follow sequences coding for export signals. At the same time, restriction enzyme analysis of such insertions specifies the orientation of the Tn*phoA* and thus of the gene itself. This approach has been used to characterize genes coding for two periplasmic acid phosphatases (3, 36) and a periplasmic protease, DegP (43), of *E. coli*.

Chromosomal genes. A major feature of the Tn*phoA* system is that it can be used to preferentially distinguish those chromosomal genes which code for cell envelope or secreted proteins; such genes would be detected as ones yielding active fusions. Then, among this subset of genes, one can screen for those genes involved in a common process or those regulated by a common stimulus. For instance, the approach has been used to detect genes of the bacterium *Rhizobium meliloti* coding for cell envelope or secreted proteins which are required for plant symbiosis (25).

Cell envelope proteins subject to a common regulatory stimulus can be recognized by examining a collection of chromosomal *phoA* fusions for their response to a change in medium or growth conditions. Eight genes subject to osmoregulation which had not previously been described were detected in this way (18).

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Bacterial virulence genes. Scanning the chromosome with Tn*phoA* has been used in pathogenic microbes to define new genes required for virulence (10, 23, 33, 35, 46). This has proved to be a particularly powerful approach since envelope and extracellularly secreted proteins are the ones most commonly involved in bacterial virulence. Thus, the genes for the major subunit of a pilus colonization factor and for a highly immunogenic outer membrane protein were identified from an analysis of 40 PhoA$^+$ chromosomal Tn*phoA* inserts in *Vibrio cholerae* (46). Further, screening for Tn*phoA* fusions that are regulated by the same environmental signals which control virulence factor expression enriches for mutations in virulence genes that are part of the same regulon. For example, more than 80% of Tn*phoA* fusions to genes of *V. cholerae* that are coordinately regulated with cholera toxin show virulence defects (35). In *Bordetella pertussis*, more than 10% of all PhoA$^+$ fusions are in genes coordinately regulated with pertussis toxin (23). Where a regulatory gene for virulence is known, one can screen for Tn*phoA* fusions that depend on the regulator for expression and, therefore, are likely to define genes for cell envelope or secreted virulence factors. In this way, a gene (*pagC*) involved in virulence of *Salmonella typhimurium* was identified solely by virtue of its response to the virulence regulator encoded by the *phoP* locus (33).

IDENTIFYING EXPORT SIGNALS IN PROTEINS

A set of alkaline phosphatase fusions can help locate an export signal in the sequence of an envelope protein, since the signal must be present in the shortest fusions showing alkaline phosphatase activity. This strategy is illustrated in an analysis of the pilin protein of *Pseudomonas aeruginosa* (44). The properties of different-length alkaline phosphatase hybrids showed not only that the export signal was present at the N terminus of the protein but also that it included amino acid residues from both a leader peptide normally removed after secretion and the N-terminal region of the mature protein.

Alkaline phosphatase fusions have also been used to identify export signals in cytoplasmic membrane proteins lacking cleavable signal sequences. For a number of such proteins, the shortest active hybrids contain alkaline phosphatase attached close to the end of the first presumptive transmembrane segment of the protein (22, 28). The first transmembrane segment (together with the region amino terminal to it) evidently functions as an internal signal sequence to promote membrane insertion.

ANALYSIS OF MEMBRANE PROTEIN TOPOLOGICAL STRUCTURE

A fundamental element of the structure of a membrane protein is its transmembrane topology, the disposition of segments of the polypeptide chain within or to one side or the other of the membrane. The analysis of alkaline phosphatase fusions can help reveal the topology of cytoplasmic membrane proteins, since the sites at which attached alkaline phosphatase has high enzymatic activity normally correspond to periplasmic domains of the membrane protein (28). This enzymatic activity is due to export of the alkaline phosphatase moiety of the hybrid protein to the periplasm. Cytoplasmic domain fusions generally show greatly reduced activities. The low enzymatic activity seen with cytoplasmic fusions is accounted for entirely by a small amount of export of alkaline phosphatase. Some caution should be used in interpreting such studies, since the alkaline phosphatase fusions lack membrane protein sequences C terminal to the fusion joint. Topological models derived by this approach should be considered as tentative. Nevertheless, the approach has yielded the correct topology for several proteins of known structure (6, 22, 27, 38; J. Cadamia and C. Manoil, unpublished results). These findings suggest that the sequences N terminal to a site in a membrane protein are often sufficient to determine its cytoplasmic or periplasmic disposition. As a result, the alkaline phosphatase fusion approach has been widely used for membrane protein topological analysis (2, 4, 6, 15, 20, 22, 27, 28, 34, 38, 47, 48).

Through the use of fusions, the genetic analysis of membrane protein topology has been extended to proteins other than alkaline phosphatase. A fusion system based on a second periplasmic protein, β-lactamase (5, 9, 11), shows properties similar to the alkaline phosphatase fusion system. The enzyme, which confers ampicillin resistance, does so only when it is exported across the cytoplasmic membrane. In a third system, β-galactosidase fusions to cytoplasmic membrane proteins have high enzymatic activity only when they are fused to the cytoplasmic domains of such proteins (12, 13). The converse identification of periplasmic sites as those with low β-galactosidase activity is complicated in that factors other than periplasmic localization can lead to low enzymatic activity (e.g., out-of-frame fusions commonly express low but detectable activity) (15; C. Manoil, unpublished results). Nevertheless, the combined use of alkaline phosphatase and β-galactosidase fusions provides positive enzymatic activity signals for both periplasmic and cytoplasmic domains in the analysis of topology. A *lacZ* fusion transposon has recently been constructed to facilitate its combined use with *phoA* fusions (C. Manoil, unpublished results). *lacZ* fusions constructed by using the new transposon can be converted into *phoA* fusions (and vice versa) by recombination, making it possible to compare the enzymatic activities of each of the two proteins attached at a particular site.

Approaches to analyzing eucaryotic membrane protein topology using gene fusions have yielded promising results. The behavior of hybrid proteins in an in vitro secretion system was used to characterize the topology of a yeast plasma membrane protein, arginine permease (1). The glycosylation pattern of different arginine permease-acid phosphatase hybrids provided a topological model for the membrane protein. Attempts to analyze topology in vivo by using the enzymatic activities of attached secretory proteins may be hindered in eucaryotic cells by the presence of factors such as BiP, a protein that binds to aberrantly folded peptides in the endoplasmic reticulum and prevents their further translocation (14). Hybrid proteins may encounter such problems. However, an approach which measured the in vivo function of the cytoplasmic protein histidinol dehydrogenase attached at different sites in a yeast endoplasmic reticulum protein (3-hydroxy-3-methylglutaryl-coenzyme A reductase) gave results consistent with a topology of the protein previously proposed from its amino acid sequence (40a). In vitro translocation studies with galactokinase fusions to the arginine permease have also been used to study the membrane topology of the latter protein (16).

An alternative approach to analyzing eucaryotic membrane protein topology is to express the protein in *E. coli* and perform the analysis by using alkaline phosphatase and β-galactosidase fusions. Eucaryotic membrane proteins have been functionally expressed in *E. coli*, implying that at

least some portion of these proteins are taking on their normal membrane structure (29, 39).

The analysis of alkaline phosphatase fusions to a membrane protein has helped define the fine structure of a topological determinant. Alkaline phosphatase fusions to the MalF protein of *E. coli* showed greater activity when positioned at the N terminus of a cytoplasmic loop than when positioned at the C terminus of the same loop (4). This result implies that the cytoplasmic sequences of the loops themselves contribute to the membrane-anchoring function of the transmembrane segments preceding them. Mutational analysis of a cytoplasmic loop indicated that positively charged amino acids in these regions play a key role in the anchoring function and are thus topogenic determinants (D. Boyd and J. Beckwith, Proc. Natl. Acad. Sci. USA, in press).

NOVEL USES OF ALKALINE PHOSPHATASE FUSIONS

Alkaline phosphatase can be used to assess the role of a periplasmic domain in the function of a protein. This use is illustrated in the analysis of ToxR protein, an activator of cholera toxin transcription (34). ToxR protein appears to consist of a cytoplasmic N-terminal domain, a single transmembrane segment, and a periplasmic C-terminal domain. Hybrid ToxR-PhoA proteins with nearly all of the periplasmic domain replaced by alkaline phosphatase maintained the transcription activation function, demonstrating that the ToxR protein periplasmic domain is not required for this activity. Further, cells producing the ToxR-PhoA hybrid protein failed to regulate toxin expression normally, implying that the C-terminal domain may function to modulate the transcription activation function of the N-terminal domain.

Alkaline phosphatase fusions have also been used to isolate mutants in which the physiology of the periplasm is affected. Hybrid proteins carrying periplasmic alkaline phosphatase are often subject to proteolysis, releasing a breakdown product about the size of alkaline phosphatase itself. Use of a simple plate screen, the blue halo technique, for mutant colonies failing to generate this breakdown product from a membrane-bound hybrid yielded mutants defective in a periplasmic protease (42). The protease appears to be active in degrading a wide variety of improperly folded periplasmic proteins. Mutant cells grew poorly at elevated temperatures, suggesting a role of proteolysis in survival under these conditions (43).

Other potential uses of the alkaline phosphatase fusion system include (i) genetic studies of special classes of export signals which promote protein secretion beyond the outer membrane (8, 37; L. Gilson and R. Kolter, personal communication); (ii) selection for mutants which enhance protein export for specific proteins (30); and (iii) detection of the subcellular locations of specially localized proteins by using electron microscopic techniques and stains or antibodies to alkaline phosphatase (7).

SUMMARY

Alkaline phosphatase fusions allow genes to be identified solely on the basis of their protein products being exported from the cytoplasm. Thus, the use of such fusions helps render biological processes which involve cell envelope and secreted proteins accessible to a sophisticated genetic analysis. Furthermore, alkaline phosphatase fusions can be used to locate export signals. Specifying such signals is an important component of studies on the structure of individual cell envelope proteins.

The basis of the alkaline phosphatase fusion approach is the finding that the activity of the enzyme responds differently to different environments. Thus, the activity of the fusion protein gives evidence as to its location. This general approach of using sensor proteins which vary in their function, depending on their environment, could be extended to the study of other sorts of problems. It may be that certain enzymes will provide an assay for localization to a particular subcellular compartment, if the environment of the compartment differs from that of others. For instance, the lysosome is more acidic than other intracellular organelles. A gene fusion system employing a reporter enzyme that could show activity only at the pH of the lysosome could allow the detection of signals determining lysosomal localization. Analogous types of enzymes may be used as probes for other subcellular compartments.

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ADDENDUM IN PROOF

In a recent paper (38), we have presented a detailed description of the types of experiments necessary to utilize the alkaline phosphatase fusion approach to membrane protein topology.

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