

Molecular Biology II: Protein function

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Abstract

While DNA may be viewed as the blueprint for the body, and mRNA/protein the component parts made from this blueprint, it is only when proteins are allowed to interact with each other and their surroundings that true biological complexity is achieved. Thus, while it is informative to study transcriptional control and mRNA transcript levels it is important to take this into the context of the total environment the encoded proteins are expressed in and how they interact with each other. At the very simplest level, expression of a ligand-activated receptor is of no biological consequence if the ligand is never present in the system, or the target for the activated receptor expressed in that cell type. It is thus important to be able to study these interactions to enable a comprehensive understanding of the biological interactions that underlying any particular phenotype. This article will outline the basic technologies to both visualise protein localisation and interaction between co-localised proteins. In addition, the manipulation of protein levels, both in vitro and in vivo, will be described, as this provides an important tool for the further examination of protein functionality within biological systems.

Protein Quantitation

The initial step in any investigation of protein expression is to be able to accurately determine the level of expression. This is particularly important as mRNA levels do not always correlate with protein levels and hence measurement at the mRNA level alone may be misleading (Greenbaum et al. 2003). The simplest way of measuring protein expression is through a Western blot (Burnette 1981). In this technique samples are separated by size by electrophoresis through a polyacrylamide gel (PAGE), with smaller fragments moving faster and hence further in a given time. As proteins can adopt three dimensional structures, which could impact on their migration rate through the gel, it is usual to add the surfactant sodium dodecyl sulphate (SDS) to the gel and hence the procedure is often referred to as SDS-PAGE. Once the samples have been separated by size it is necessary to identify the protein of interest from within the multitude of proteins separated on the gel. To achieve this, proteins are first transferred from the polyacrylamide gel to a more robust support, usually nitrocellulose or PVDF membrane, which is then 'blocked' with an albumin mixture (usually powdered milk) to prevent non-specific binding of the antibody in the next stage. Following blocking an antibody specific to the target protein is incubated with the sample, followed by several washes to remove non-specifically bound antibody. Finally, the antibody is detected, usually through the use of colourimetric or fluorometric substrates to enzymes linked to the probe antibody. This procedure will result in a band corresponding to your target protein, with the intensity of the band being correlated to the amount of protein present. Due to the sometimes questionable specificity of antibodies, it is usual to include a recombinant expressed version of the target protein in one lane, thus providing proof that the band is specific for the target proteins. Another issue in this procedure is how to control for even loading of samples into the wells, for if this is not achieved then differential band strengths may be observed as an artefact. Blots are 'stripped' using the surfactants SDS and tween-20 and then reprobbed with an antibody for a general protein such as actin; this can then be used to normalise numbers for each lane and ensure a robust quantitation (Plant et al. 2009).

As mentioned, one issue with Western blotting is the quality of the antibody, which often shows considerably less specificity and sensitivity than the probes used to analyse mRNA levels. However, this disadvantage must be weighed against the increased biological relevance of the answer, including the fact that phospho-specific antibodies are now available for many proteins; as phosphorylation is a central post-translational mechanism for switching proteins on and off this means that it is possible to not only measure the level of a protein but also distinguish between active and inactive forms (Abate et al. 1993).

Protein:Protein Interaction

It is often necessary for two, or more, proteins to interact before they can exert their biological effect. Such effects can include the interaction of dimerisation partners prior to DNA binding (Abate et al. 1993), interaction of proteins in signalling cascades (Budihardjo et al. 1999), or interaction of proteins to permit sub-cellular localisation (Plant et al. 2006). It is thus necessary to examine the interaction of proteins to fully understand their biological functioning, and several techniques exist to permit this.

Perhaps the simplest technology is co-localisation, which works on the premise of guilt by association. The sub-cellular localisation of two proteins is determined, either using an antibody-based technique similar to Western blotting but undertaken on whole cells (immunocytochemistry), or by creating plasmids containing each protein fused to a fluorophore, meaning the target protein will emit light of a characteristic wavelength. Following transfection into a cell line if the signal for the two proteins overlap then it can be assumed that they are in close proximity within the cell and may be interacting (Huang et al. 2004). However, this is not proof positive and further steps are required to prove interaction. Whereas co-localisation experiments show that proteins are within micrometers (10^{-6} m) of each other, fluorescence resonance energy transfer (FRET) determines co-localisation within angstroms (10^{-10} m). FRET uses a similar approach to co-localisation in that both proteins are tagged with a fluorophore, but in this instance the fluorophores are chosen such that

the emission spectrum of one fluorophore (A) overlaps the excitation spectrum of the other (B). If the proteins localise separately within the cell then excitation of fluorophore A will result in the characteristic light emission of A, and the same for fluorophore B. However, if the proteins are within 100Å, which almost certainly means that they are interacting, then excitation of fluorophore A will result in emission of light characteristic of fluorophore B as the light energy is transferred (Hillisch et al. 2001). Finally, interaction can be positively proven through co-precipitation assays: This assay is very similar to the chromatin immunoprecipitation (ChIP) assay used to identify protein:DNA interactions, described in the preceding article, and relies on the precipitation of one protein with a specific antibody, thus co-precipitating any interacting proteins as well (Shaw 2001).

Altering protein expression levels

The examination of protein levels by Western blotting, and the interaction of proteins in the cellular context through co-localisation, FRET and co-immunoprecipitation lend great support to understanding the roles of proteins within the cell. However, a useful technology to really explore the functionality of proteins is the ability to artificially alter their expression levels. Over-expressing or knock-down of protein expression can help determine what processes in a cell a protein is involved in. To achieve this manipulation different, but related, technologies are used depending on whether you wish to alter protein expression in cell lines (in vitro) or in whole animals (in vivo).

Altering Protein levels in vitro

To increase the level of a protein in a cell line is a relatively simple task. First, the DNA encoding the protein of interest is cloned into an expression plasmid, a circular DNA that can be transfected into the cell line of choice for expression. This plasmid will then produce the protein encoded by the DNA, either constitutively or following the addition of stimulating chemical. Assays can then be undertaken to examine the effect of this over-expression on any particular biological process, showing how the target protein impacts on this process (El-Sankary et al. 2000). Knock down of

protein expression is achieved through a similar methodology, except that instead of transfecting an expression plasmid into the cell line small interfering RNAs (RNAi) against the target protein are instead transfected into the cell line: RNAi mimics naturally occurring micro RNAs, which cause mRNA degradation and are used by the body to regulate mRNA transcript pool size (Pasquinelli 2002). Transfection of RNAi into a cell line will degrade the mRNA transcript pool for the target protein and ultimately lead to reduction in protein levels. However, it is important to note that as protein levels will persist for some time after the mRNA transcript pool has been depleted, and that depletion is seldom 100%, it is important that Western blotting is used to show that suppression of protein levels has truly been achieved (Plant et al. 2009).

Altering Protein levels in vivo

Alteration of protein levels in vivo is considerably more complex than in vitro. Although it is possible to use over-expression plasmids and RNAi as described previously these approaches are technically demanding and seldom 100% successful. By far and away the more common approach is the production of transgenic animals, predominantly mice. The reason for mice being the animal of choice for mammalian transgenics reflects their ease of husbandry, relatively fast generation time and, most importantly, ability to harvest embryonic stem (ES) cells. ES cells are derived from the inner cell mass of a blastocyst and are the totipotent cells that go on to form the foetus. These cells can be easily isolated from mice and if grown on a differentiation inhibiting medium can be cultured almost indefinitely. Once these cells have been manipulated to contain the requisite genetic material (described in the next paragraph) they can be injected back into the core of a blastocyst, which in turn can be implanted into a pseudopregnant female mouse. The resultant pups will be hemizygous chimeras as they will be derived from both the inserted, modified, ES cells and those ES cells that were already present in the blastocyst. Further breeding of the chimaeric transgenic pups with wild type mice will result in fully transgenic mice, where every cell contains the transgene; if

this is present on only one sister chromosome the mice are heterozygous, but if both sister chromosomes contain the transgene then the animal is homozygous (Bradely et al. 2001).

The process for inserting a transgene into genomic DNA is relatively simple and can be carried out in a non-targeted or targeted fashion. For non-targeted integration the exact location in which the transgene inserts into the genomic DNA is not controlled. This has the advantage that no prior knowledge is required about the integration site and is common for the integration of plasmids to over-express a protein (knock-in). In addition, a gene encoding antibiotic resistance is also included in this integration region, thus allowing selection of those ES cells where integration has been successful. However, the lack of control over the integration site can lead to problems if the transgene, such as integration into a region of compressed chromatin, leading to reduced expression of the transgenic protein. Alternatively, the transgene could integrate in to another gene, disrupting its expression in the process, and thus produce a phenotype that is difficult to interpret (Bradely et al. 2001). By comparison, targeted integration provides total control over the integration site and has the added advantage that this now allows disruption of protein expression from genes already present in the genome (knock-out). For targeted integration the targeting plasmid is designed such that it includes two regions of DNA that exactly match the genomic DNA; these then act as the points for homologous recombination and integration to occur (Figure 1). In between these two regions can be included any DNA that you wish to incorporate into the genome, such as the over-expression sequence and the antibiotic resistance gene. To create a knock-out it is simply a matter of designing the homologous regions so that they interrupt the genomic copy of the target gene (Figure 1)(Xie et al. 2000). In addition to an antibiotic resistance gene for integration, the targeting plasmid usually includes a second marker, thymidine kinase (TK). As the TK gene is outside of the region subject to homologous recombination then if targeting is successful it will not be included in to the genomic DNA; if however, the targeting plasmid integrates randomly then the TK will also integrate. A simple TK assay can hence be used to determine the correct targeting and integration of the construct into the genomic DNA.

Conclusions

The ability to study protein function within a living cell, both in vitro and in vivo, is central to our ability to understand how the body functions. Such studies will allow us to understand the biological interactions that occur within cells and how these alter under different stimuli. Finally, the ability to alter expression levels of proteins, either increasing or decreasing them, adds the tantalizing prospect of being able to recreate disease conditions, thus providing further insights into the pathogenesis of disease and novel potential therapies.

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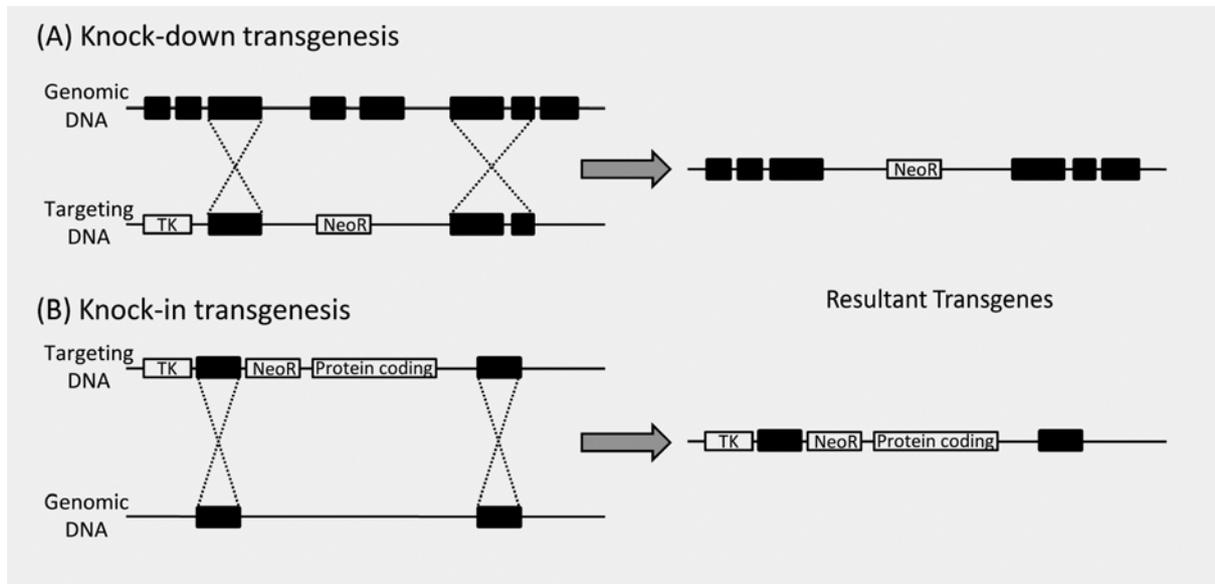


Figure 1: Construction of targeted transgenes: (A) To create a transgenic knock-out the targeting constructs are designed to match the exons within the target gene. These then undergo homologous recombination and integrate the selection marker, antibiotic resistance, into the genomic DNA. (B) To create a knock-in the targeting constructs are designed to integrate both the antibiotic resistance and coding region for the protein to be expressed into a region of DNA that will allow expression, but not disrupt any other genes. In both cases inclusion of antibiotic resistance (NeoR) and a selectable marker (TK) are used to ensure specific integration of the targeting construct.