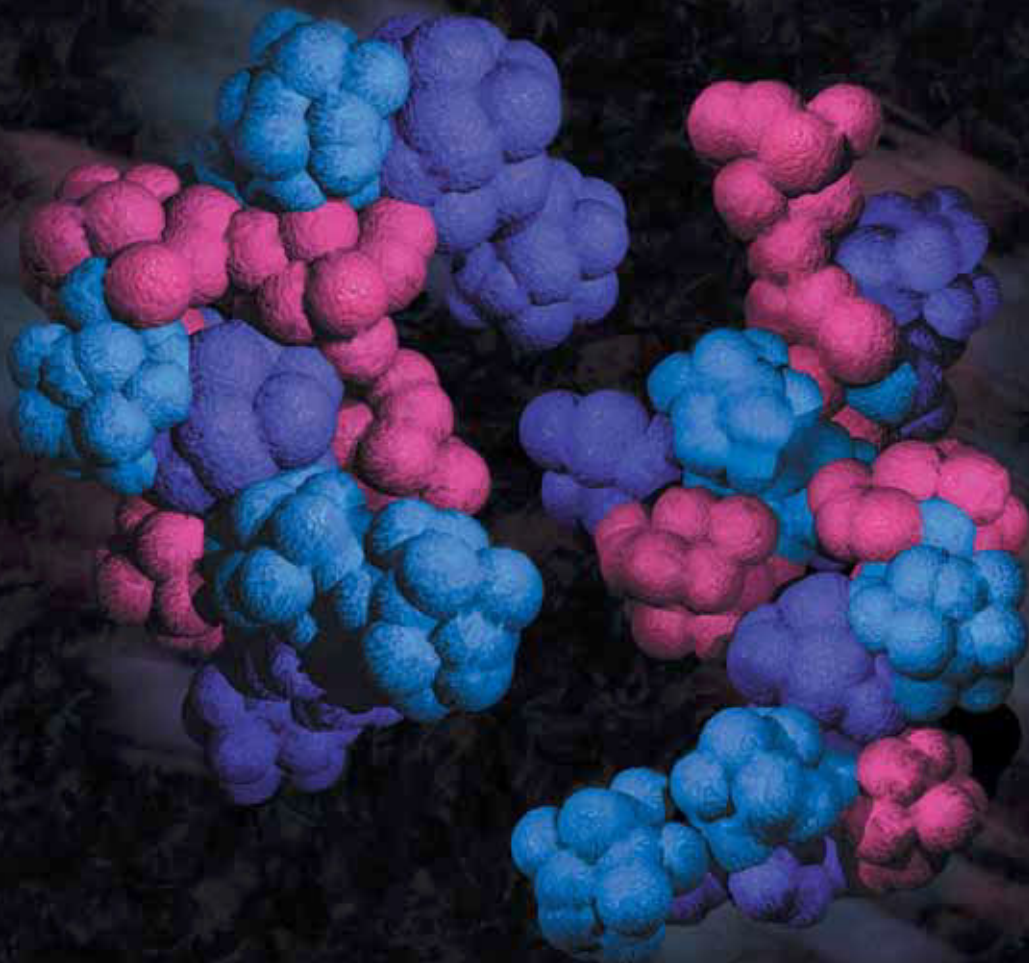


CHAPTER

# 2

**About the Image:**

*This illustration of protein-protein interactions shows the complementary fit of two protein molecules, such as a ligand and its receptor molecule on the cell's surface.*



## Chapter Two: Protein-Protein Interactions

Contents	Page
Introduction .....	8
Fusion Tag Approach .....	9
Immunoprecipitation .....	10
Far Western Analysis .....	11
Isolation of Protein Complexes by Capture of Biotinylated Lysine Residues .....	11
Protein Folding, Chaperonins and Luciferase .....	12
Real-Time Translation/Folding Assays .....	12
Macromolecular Assembly and Frameshifting.....	12

## References

1. Boyd, J. *et al.* (1995)  
*Oncogene* **11**, 1921.

## Introduction

A popular current application of the coupled eukaryotic systems is detection of protein-protein interactions. Often researchers will use the in vitro approach to verify or confirm in vivo results, such as those obtained using the yeast two-hybrid approach (1). To define the region of protein-protein interaction, usually a series of deletion constructs or occasionally specific point mutants are synthesized in vitro and compared to wildtype, full-length proteins.

### TO ORDER

**Phone**  
1-800-356-9526  
**Fax**  
1-800-356-1970  
**Online**  
[www.promega.com](http://www.promega.com)



**Promega**  
[www.promega.com](http://www.promega.com)

**Fusion Tag Approach**

Gene products expressed *in vivo* as fusion or “tagged” proteins can be used to detect protein-protein interactions or as an alternative to antibody detection. Radioactive proteins can be used as probes to detect interactions with suspected protein partners that have been expressed in *E. coli* as GST- (glutathione-S-transferase), His- or epitope-tagged fusion proteins (2). [<sup>35</sup>S]methionine-labeled proteins can be synthesized using coupled *in vitro* reactions from either full-length cDNAs or deletion mutants. The fusion proteins can be bound to an affinity matrix along with the radioactive proteins with which they interact (3,4,5). The bound radioactive proteins are then eluted and analyzed by SDS-PAGE or Western analysis (Figure 1; 5).

Alternatively, a nonradioactive approach may be used; the protein is labeled with biotinylated lysine (e.g., Transcend™ Biotinylated tRNA) and combined with a GST-tagged protein. The biotinylated protein is detected by means common to Western blotting (6,7).

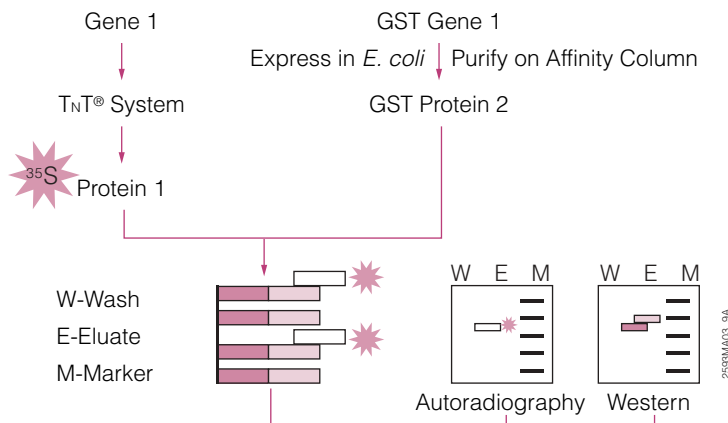
This fusion tag approach has been used to study receptor-mediated control of apoptosis. Binding of Fas ligand (FasL) or anti-Fas antibody to Fas (APO-1/CD95) receptor, or binding

of tumor necrosis factor (TNF) to the TNF receptor (TNFR-1) rapidly induces cell death by an as yet undetermined mechanism. A unique cytoplasmic motif present in both TNFR-1 and Fas, the “death domain,” is necessary for induction of cell death. The death domain is the site of protein-protein interaction. The primary function of FasL and TNF, as recently postulated, is to mediate receptor aggregation (8). Therefore, a critical step is to identify proteins that bind directly to the cytoplasmic death domains of these receptors.

FADD is a Fas-associated protein containing a novel death domain that was identified by Chinnaiyan *et al.* (2). Using Promega’s TnT® T7 Coupled Reticulocyte Lysate System<sup>(a,b,c,e)</sup> (Cat.# L4610), these researchers synthesized <sup>35</sup>S-labeled FADD *in vitro* from a modified expression vector. Labeled FADD was incubated with wildtype and mutant GST-Fas fusion proteins as well as a GST-TNF fusion protein. Figure 2A shows the five GST fusion proteins used in the binding assays. Figure 2B presents the results of the binding experiment, illustrating that FADD binds only the wildtype Fas construct and the mutant Fas-FD5 construct. The Fas-FD5 construct encodes a protein with enhanced apoptotic activity compared to that encoded by the wildtype Fas.

**References (continued)**

2. Chinnaiyan, A.M. *et al.* (1995) *Cell* **81**, 505.
3. Cowell, I. and Hurst, H. (1996) *Nucl. Acids Res.* **24**, 3607.
4. Sharp, T.V., Witzel, J.E. and Jagus, R. (1997) *Eur. J. Biochem.* **250**, 85.
5. Jagus, R. and Beckler, G.S. (1998). Overview of eukaryotic *in vitro* translation and expression systems. *Current Protocols in Cell Biology* 11.1.1–11.1.13. Copyright © 1998 by John Wiley & Sons, Inc. Reproduced by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.
6. Pei, L. (1999) *J. Biol. Chem.* **274**, 3151.
7. Chen, W. and Pei, L. (2000) *J. Biol. Chem.* **275**, 19422.
8. Cleveland, D.L. and Ihle, J.H. (1995) *Cell* **81**, 479.



**Figure 1. The study of protein-protein interactions using the TnT® Systems (5).** This schematic shows translation of one protein with radioactive [<sup>35</sup>S]methionine in a TnT® System reaction. Large amounts of the suspected partner protein are expressed and purified from *E. coli*. A fusion tag (most commonly GST) is incorporated into this second protein to facilitate purification and subsequent capture steps. After the GST fusion protein is immobilized on glutathione-agarose, it is mixed with the protein produced in the TnT® reaction. The agarose is washed to remove unbound protein and the remaining bound proteins are eluted and analyzed on a gel. This technique allows quantitative measurement of the protein-protein interactions for both wildtype and mutant proteins and is often used to verify the *in vivo* results obtained from yeast two-hybrid experiments.

**TO ORDER**  
**Phone**  
 1-800-356-9526  
**Fax**  
 1-800-356-1970  
**Online**  
[www.promega.com](http://www.promega.com)



References (continued)

9. Benedict, C.M. and Clawson, G.A. (1996) *Biochemistry* **35**, 11612.
10. Leng, P., Brown, D.R. and Deb, S. (1995) *Int. J. Oncol.* **6**, 251.
11. Sif, S. and Gilmore, T.D. (1993) *J. Virol.* **67**, 7612.
12. Rice, N.R., MacKichan, M.L. and Israel, A. (1992) *Cell* **71**, 243.
13. Mercurio, F. *et al.* (1993) *Genes Dev.* **7**, 705.

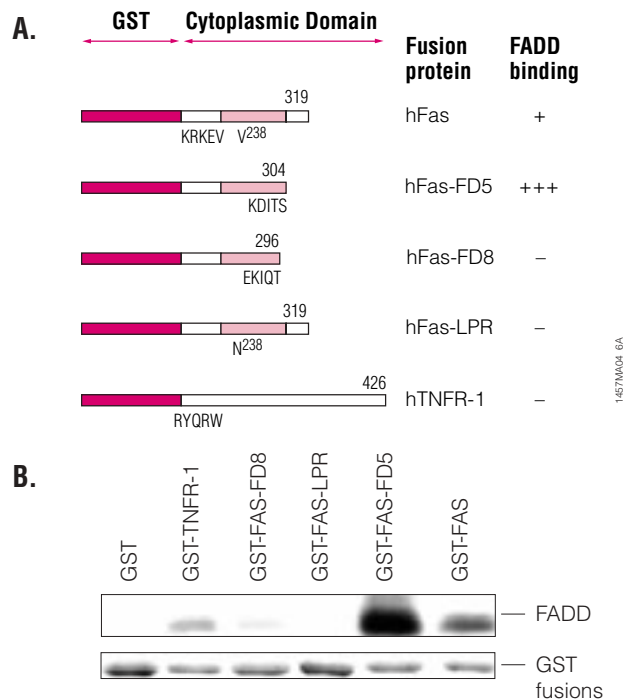
Immunoprecipitation (5)

This approach utilizes antibodies against a particular antigenic domain to detect a radioactive fusion partner. For example, an influenza hemagglutinin (HA) epitope incorporated in the carboxyl terminus of an in vitro expressed protein can be immunoprecipitated using anti-HA antibodies (9). Alternatively, if an antibody against one of the partners is available, then co-immunoprecipitation can be used for detection (4, 10). Again, the bound radioactive proteins are then eluted and analyzed by SDS-PAGE or Western blot analysis. A variation of this type of analysis uses in vitro expression of several proteins simultaneously in a coupled system. The relative protein expression levels can be controlled by varying the amount of each DNA construct. For instance, experiments using cDNAs for chicken NF- $\kappa$ B p105, NF- $\kappa$ B p100, c-Rel, and v-Rel, cotranslated in vitro, followed by protein complex detection by immunoprecipitation with specific antiserum, show that one of the demonstrated complexes from v-Rel-trans-

formed spleen cells can be reconstituted in vitro (5, 11).

Proteins in the NF- $\kappa$ B transcriptional activator family act as tertiary messengers, transducing signals from the environment to the nucleus of the cell. NF- $\kappa$ B resides in the cytoplasm as an inactive complex consisting of heterodimeric DNA-binding subunits sequestered by an inhibitor (I- $\kappa$ B). When an appropriate signal is received, I- $\kappa$ B is thought to be phosphorylated and then dissociates from the DNA-binding subunits, allowing the subunits to translocate to the nucleus and bind their target genes.

In one pathway, the NF- $\kappa$ B precursor proteins p105 and p98 can form heterocomplexes with both the NF- $\kappa$ B subunit p65 (RelA) and the proto-oncogene product c-Rel. This results in the retention of these heterocomplexes in the cytoplasm (12, 13). When p105 or p98 is proteolytically processed to yield p50 or p55, the DNA binding subunits are released from the heterocomplexes and are free to translocate to the nucleus or to interact with I- $\kappa$ B.



**Figure 2. Specific interaction of GST-Fas and GST-Fas-FD5 with in vitro translated FADD and FADD expressed in transfected 293T cells.** **Panel A:** Schematic representation of the GST fusion proteins containing the cytoplasmic domains of Fas, Fas mutants and TNFR-1. Amino acid residues are given for selected junctions, and numbering is based on the mature form of the receptor. The lymphoproliferation (*lpr*) mutant of Fas is represented ( $V^{238} \rightarrow N^{238}$ ). Gray shading represents the death domain of Fas. Binding of FADD to the various GST fusion proteins is depicted at the right and is based on data from Panel B. **Panel B:** Interaction of in vitro translated,  $^{35}$ S-labeled FADD with various GST fusion proteins immobilized on glutathione-Sepharose® beads. After the beads were washed, retained FADD protein was analyzed by SDS-PAGE and autoradiography (upper panel). The gel was Coomassie®-stained, and the bands representing the various GST fusion proteins were aligned to show equivalency of loading.

TO ORDER

Phone  
1-800-356-9526  
Fax  
1-800-356-1970  
Online  
www.promega.com



Promega  
www.promega.com

DiDonato and Karin (14) used immunoprecipitation to demonstrate that the p98 and c-Rel proteins are capable of interacting in vitro. In the experiment shown in Figure 3, a c-Rel-specific antibody was used to immunoprecipitate c-Rel from TNT® reactions expressing either p98, c-Rel or both of these proteins. p98 was coprecipitated when expressed in the presence of c-Rel (lanes 1–3) but not when expressed alone (lane 5).

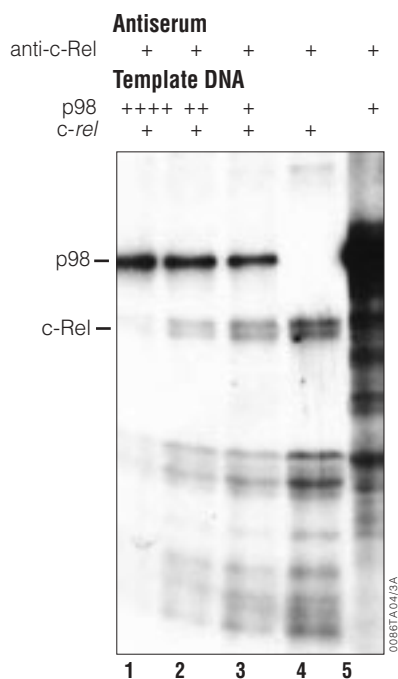
### Far Western Analysis (5)

A direct detection method for identifying protein-protein interactions after transfer of proteins from polyacrylamide gels to a membrane has been termed “Far Western” analysis. In this approach, radioactive proteins are synthesized in vitro and then used as probes to directly detect binding to membrane-bound, renatured proteins (15).

### Isolation of Protein Complexes by Capture of Biotinylated Lysine Residues (5)

One drawback of fusion protein techniques is the requirement to make the fusion protein con-

struct. Another approach that obviates the need for the development of novel constructs is to incorporate non-natural amino acids, such as biotinylated lysine residues into the in vitro translated proteins. This approach will work for any gene containing lysine codons. The incorporation of non-natural amino acids does not significantly alter the efficiency of polypeptide synthesis, and often incorporation of biotinylated lysines does not affect the function of the protein (16). This approach has been used to develop a novel method for capturing protein complexes that associate with biotinylated Rab5, a member of the Rab family of GTP-binding proteins (17). The ability to bind biotinylated lysine tightly to streptavidin-linked agarose can be utilized to capture in vitro synthesized biotinylated Rab5. A promising and potentially powerful modification of the biotinylated-lysine capture technique proposes using photocleavable (PC)-biotin for the detection and gentle purification of in vitro generated polypeptides (18). For example, the capture of the PC-biotin nascent polypeptides using streptavidin-coated magnetic beads has been described. After a short exposure to UV light, the nascent



**Figure 3. Protein-protein binding studies using proteins co-expressed in the TNT® Wheat Germ System.** <sup>35</sup>S-labeled c-Rel and p98 were produced in 25µl TNT® Wheat Germ Extract<sup>(a,b,c,e)</sup> reactions containing 1.5µg of *c-rel* template DNA (lanes 1–4) and 1.0µg, 0.5µg, 0.25µg or 0.25µg of p98 template DNA (lanes 1, 2, 3 and 5, respectively). A 4µl aliquot from each reaction was analyzed by immunoprecipitation with anti-c-Rel antiserum as described in reference 10 and then analyzed by 10% SDS-PAGE. For the gel shown in this figure, lanes 1–3 were loaded to contain equivalent amounts of p98. The immunoprecipitation results were detected by autoradiography after 16 hours at –70°C.

### References (continued)

- DiDonato, J.A. and Karin, M. (1993) *Promega Notes* **42**, 16.
- Johnston, S., Yu, X.M. and Mertz, J.E. (1996) *J. Virol.* **70**, 1191.
- Beckler, G. and Hurst, R. (1993) *Promega Notes* **43**, 24.
- Sanford, J.C. *et al.* (1995) *J. Biol. Chem.* **270**, 26904.
- Rothschild, K.J., Sonar, S.M. and Olejnik, J. (1997) U.S. Pat. No. 5,643,722.

### TO ORDER

Phone  
1-800-356-9526

Fax  
1-800-356-1970

Online  
[www.promega.com](http://www.promega.com)



**Promega**  
[www.promega.com](http://www.promega.com)

### References (continued)

19. Olejnik, J. *et al.* (1995) *Proc. Natl. Acad. Sci. USA* **92**, 7590.
20. Olejnik, J., Krzymanska-Olejnik, E. and Rothschild, K.J. (1998) *Meth. Enzymol.* **291**, 135.
21. Bazzoni, G. *et al.* (2000) *J. Biol. Chem.* **275**, 20520.
22. Frydman, J. *et al.* (1994) *Nature* **370**, 111.
23. Frydman, J. and Hartl, F.U. (1996) *Science* **272**, 1497.
24. Kolb, V.A., Makeyev, E.V. and Spirin, A.S. (1994) *EMBO J.* **13**, 3631.
25. Makeyev, E.V., Kolb, V.A. and Spirin, A.S. (1996) *FEBS Lett.* **378**, 166.
26. Sakalian, M. *et al.* (1996) *J. Virol.* **70**, 3706.

polypeptide was released (70–95% efficiency) in native form with no remaining “tags” (18–20). In another procedure, the ZO-1 protein and its role as a tight junction component was studied by using the TNT<sup>®</sup> Coupled Reticulocyte Lysate System<sup>(a,b,c,e)</sup> and Transcend<sup>™</sup> Biotinylated tRNA (21).

### Protein Folding, Chaperonins and Luciferase (5)

In vitro expression is increasingly being used to understand the nature of sequential chaperonin interactions required for protein folding. Researchers in this field have combined the advantages of in vitro expression with the power of instantaneous reporter gene product assays. The folding of polypeptides emerging from ribosomes has been analyzed using firefly luciferase as a model protein (22,23). The growing polypeptide interacts with a specific set of molecular chaperones, including Hsp70, the DnaJ homologue Hsp40 and the chaperonin TRiC. The ordered assembly of these components on the nascent chain forms a high molecular mass complex that allows the cotranslational formation of protein domains and the completion of folding once the chain is released from the ribosome.

### Real-Time Translation/Folding Assays (5)

A novel approach has been developed using a wheat germ system in which the components for the luciferase enzymatic assay have been added directly to the translation reaction and monitored continuously in real time (24). In order to demonstrate that luciferase exhibits cotranslational folding, a comparison was made of the activity of translation products produced from wildtype mRNAs with those produced from mutant mRNAs lacking stop codons to prevent release of the polypeptide from the ribosome. Luciferase was shown to be completely folded and fully active immediately upon release from

the ribosome (24). However, no luciferase activity was observed, while full-length luciferase remained attached to the ribosome as a peptidyl-tRNA, probably because the C-terminal portion of the enzyme is masked by the ribosome and/or ribosome-associated proteins. The investigators demonstrated that the ribosome-bound enzyme acquires enzymatic activity when its C-terminus is extended by at least 26 additional amino acid residues (25). The results demonstrate that the acquisition of the final native conformation by a nascent protein can occur as the protein is being synthesized and that folding does not require release of the protein from the ribosome.

### Macromolecular Assembly and Frameshifting (5)

Many in vivo translational control mechanisms are faithfully replicated in vitro. In addition, a variety of macromolecular complexes can be expressed and properly assembled in vitro. For example, an in vitro synthesis and assembly system for the prototypical type D retrovirus, Mason-Pfizer monkey virus (M-PMV), has been developed. This system uses rabbit reticulocyte reactions expressing M-PMV Gag precursor polyproteins as the result of two ribosomal frameshift events (26). The frameshift efficiency in vitro is identical to that observed in vivo. These polyproteins assemble to form immature retrovirus capsids indistinguishable from those formed in the host cell cytoplasm. More importantly, this system can be utilized for the analysis for potential inhibitors of retrovirus assembly with the use of anti-Gag antibodies.

#### TO ORDER

Phone  
1-800-356-9526

Fax  
1-800-356-1970

Online  
[www.promega.com](http://www.promega.com)



**Promega**  
[www.promega.com](http://www.promega.com)