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Methods to map protein interactions in mammalian cells: different tools to address different questions



Abstract

In the post-genome era, functional annotation of the predicted gene-sets will be one of the most important upcoming challenges. So-called interactome analysis positions a protein in its subcellular environment by mapping its interaction partners. Such interaction maps are essential for an accurate insight into protein function since many cellular processes are organised to operate in protein complexes. These assemblies have dynamic structures and can interact with each other, two properties which are often controlled by regulated protein

expression and modification. Various methods exist to unravel protein interaction circuitries, which can be roughly divided into biochemical and genetic strategies. In this review we focus on the different strategies to study protein-protein interactions in living mammalian cells. Recently developed analytical and screening methods are also addressed.

Full text

ABBREVIATIONS

BRET: bioluminescence resonance energy transfer; DHFR: dihydrofolate reductase; FACS: fluorescence-assisted cell sorting; FKBP: FK506 binding protein; FRAP: FKBP-rapamycin binding protein; FRET: fluorescence resonance energy transfer; GEF: guanine nucleotide exchange factor; GFP: green fluorescent protein; MAPPIT: mammalian protein-protein interaction trap; RRS: Ras recruitment system; SRS: Sos recruitment system; STAT: signal transducer and activator of transcription.

INTRODUCTION

Protein interactions are essential for most cellular functions. Fundamental processes such as gene transcription, translation, protein secretion and degradation, cell cycle control, signal transduction and apoptosis all rely on the correct functioning of protein complexes. Mutations affecting protein structure often cause an altered interaction profile, which in turn can result in severe malfunction and disease. An example for this can be found in the pRB pathway which is involved in G1 to S phase transition, and which is affected in nearly all cancers. Point mutations in interaction domains, as well as absence or elevated expression of critical proteins, can result in tumour formation (reviewed in [1]).

Large-scale approaches such as complete genome sequencing, transcriptional profiling and proteomics provide a wealth of information but need to be complemented by approaches towards functional annotation of the identified proteins. Towards this end, some large-scale techniques such as overexpression of cDNAs in cell lines coupled to functional assays, subcellular localisation based on green fluorescent protein (GFP) fusions, as well as knock-out studies in lower organisms are currently used. Another strategy for

obtaining information on the function of a protein is the search for its interaction partners. The principle is based on "guilt by association" meaning that identification of the interaction partners may place a protein in an enzymatic or metabolic complex or in a signal transduction pathway [2]. The recent application of existing methods to generate interaction maps, may therefore significantly contribute to the unveiling of the biological role of a given protein. It also allows the charting of regulatory networks and can be applied to the mapping of a signal transduction pathway from the membrane to the nucleus.

Protein-protein interactions were initially studied by means of biochemical approaches such as affinity-purification and co-immunoprecipitation. An important recent improvement is the development of very sensitive mass spectrometry which, combined with data-mining in complete genome sequences, allows identification starting from very small amounts of protein. The power of this approach was recently demonstrated in the identification of a large number of protein complexes in yeast using efficient tags allowing the purification of protein complexes under mild conditions [3, 4]. The major disadvantage intrinsic to biochemical approaches remains the requirement for cell lysis. This not only causes the loss of weak, temporary interactions, but may also result in the detection of interactions between proteins that never occur inside a cell. "Sticky" proteins such as certain heat shock proteins and other chaperones are also often co-purified. The problem of false positive and false negative signals, respectively resulting from interactions that are detected, but that do not occur *in vivo* and, interactions occurring *in vivo* that are not detected, is, albeit to a variable degree, inherent to all known protein interaction detection systems.

Since the development of the first genetic screen in yeast [5, 6], insights into protein interaction networks have expanded greatly. In the so-called yeast two-hybrid system, functional reconstitution of a transcription factor *via* a protein-protein interaction results in the induction of reporter genes (for the principle refer also to fig. 1, where the equivalent approach in mammalian cells is depicted). The system is characterised by low technical requirements, low operation costs and ease of manipulation. It has been widely used by numerous research groups and has resulted in optimised protocols. The yeast two-hybrid system was also used in large-scale interaction mapping [7, 8]. However, despite its wide application and low cost, the yeast two-hybrid approach still suffers from a number of intrinsic limitations. Firstly, interactions are detected in

the nucleus of a lower eukaryote, which therefore not only requires nuclear migration, but in addition also implies a suboptimal context for protein interactions in higher organisms. Secondly, posttranslational modifications such as phosphorylation, which are essential to signal transduction processes, are hard to reproduce in yeast. Thirdly, false positive signals are often detected, creating the need for careful and tedious evaluation of the interactions. The recent comparison of interaction maps generated for yeast by two independent groups underscores these problems. Differences in the experimental protocol and tools (plasmids, reporters and yeast strains) may cause the limited overlap of only 15% observed after comparison of the interaction maps. This raises important questions on reproducibility and on the value of such interaction maps [7, 8]. Moreover, the tag-based purification approach described above, resulted in the confirmation of significantly more known interactions [9] when compared to the large-scale yeast two-hybrid approaches [3].

Some of these shortcomings can be overcome by the use of mammalian cell systems. To date a respectable number of such approaches exist, which will be discussed more in detail below. Mammalian systems are generally more complex, thus often requiring advanced cell-biology or fluorescence techniques. The different techniques available today all have specific properties and their application thus depends on the biological question(s) that needs to be addressed. In fact, the availability of more detailed structural and functional information, leads to the design of more sophisticated approaches. The range of mammalian two-hybrid systems available today nicely reflects this evolution.

Mammalian systems also offer clear advantages when applied for drug screening purposes. Some of the systems described below are well suited for the identification of lead compounds that interfere with selected protein-protein interactions. Other reverse systems rely on yeast [10] or *E. coli* [11] as a host, and are therefore sub-optimal since their membrane characteristics differ completely from mammalian cells.

ADAPTATIONS OF YEAST TWO-HYBRID STRATEGIES TO MAMMALIAN CELLS

Transcription factor complementation in mammalian cells

The first mammalian two-hybrid methods are mere adaptations of the yeast strategy. Five GAL4 transcription factor binding sites are typically fused in front

of a minimal promoter and a reporter gene. The "fishing" protein or "bait" is fused to the binding domain of the yeast transcription factor GAL4, while the interacting protein or "prey" is fused to the viral VP16 activation domain (Figure 1). The system was first used to study hetero-oligomerization between the leucine zipper domains of c-Jun and c-Fos in Chinese hamster ovary cells [12] and was rapidly expanded to detect the interaction between the CD4 receptor cytoplasmic domain and Lck, an interaction that occurs in the cytoplasm [13].

This two-hybrid approach is often applied to verify results obtained in yeast cells and is also commercially available. Some modification-dependent interactions can be monitored using this technique since binding between the signal transducer/DNA-binding protein in mammalian cells 4 (Smad4) and the melanocyte-specific gene 1 (MSG-1) can be detected after stimulation with transforming growth factor beta (TGF-beta). This interaction cannot be detected in yeast [14].

This mammalian method was also applied to obtain a limited interaction map for approximately 3,500, mouse, full length cDNAs [15]. To streamline the procedure, these authors used a simple two step PCR strategy to obtain bait and prey constructs which are preceded by a promoter sequence. These PCR amplicons encoding the protein chimeras were directly transfected into CHO cells and assayed for luciferase reporter induction. The approach allowed the detection of 145 interactions between the encoded proteins.

By fusing the GAL4 binding sites to the coding sequence for a membrane-anchored marker protein or by fusing them to the hygromycin resistance gene, it was possible to select for cells containing chimeric protein interaction pairs by using fluorescence assisted cell sorting (FACS) and survival in hygromycin-containing medium respectively [13]. To our knowledge, no successful random screening experiments have yet been published.

The mammalian Ras recruitment system

In 1997, Aronheim and colleagues described the development of a new system to detect protein-protein interactions in the cytoplasm of yeast. The Sos recruitment system (SRS) is based on the recruitment of the mammalian guanine nucleotide exchange factor (GEF) Sos to the membrane leading to activation of Ras. This results in survival of yeast cells expressing a

temperature-sensitive GEF variant (cdc25) when these were grown at the restrictive temperature. In this system, the prey is fused to a membrane targeting signal while the bait is coupled in frame to mSOS [16]. Direct recruitment of mammalian Ras to the membrane also results in survival in yeast cells and this so-called Ras recruitment system was shown to be more efficient than the SRS [17]. The latter method was also extended to mammalian cells by the use of Ras responsive reporter genes. *Via* this mammalian RRS (mRRS), Maroun *et al.* showed the interaction between the p110 and p85 subunits of phosphatidylinositol 3-kinase, and the interaction between grb-2 and human SOS in the human embryonal kidney cell line HEK293 (Figure 2; [18]). This system allows simple verification of interactions found by screening with the yeast RRS, and can, in theory, be applied to all cytoplasmic proteins provided that they do not interfere with the MAPK pathway. This requirement precludes the screening of complex cDNA libraries [18].

ENZYMATIC COMPLEMENTATION ASSAYS

Johnsson and Varshavsky pioneered the use of functional protein complementation *via* protein-interaction driven heterodimerization of discrete protein fragments. Some functional properties of enzymes require the presence of two or more protein domains. Some of these domains can be reconstituted in an intermolecular way, by means of a protein-protein interaction. Obviously the system would not work if these domains interact by themselves, and thus in some cases, additional mutations had to be introduced. Inherent problems with such protein complementation assays are the steric requirements for obtaining reconstitution. Hence, interactions that result in an incorrect orientation of the domains will not be detected. This problem can be addressed by optimising the chimeric constructs *via* different linkers, or by altering N- to C-terminal fusions or vice versa. The necessity for correct orientation in these complementation strategies will most likely cause a great number of false negatives when cDNA library screening experiments are performed. In fact, with any of the complementation assays described below, no successful cDNA hunt has been so far reported. Advantages of these assays include cell line - and often cell compartment - independence, as well as the ability to monitor some modification-dependent interactions. Four different protein complementation assays have so far been described.

Ubiquitin-based split-protein sensor

Johnsson and Varshavsky use in this complementation strategy the 76 amino acid protein ubiquitin. Ubiquitin is covalently attached to proteins destined for degradation. This ubiquitination results in the recognition by ubiquitin specific proteases initiating proteolysis. Reconstitution of two selected ubiquitin fragments (N_{ub} and C_{ub}) still leads to recognition by ubiquitin-specific-proteases. To prevent spontaneous dimerization, a mutation was introduced in the N_{ub} fragment, enforcing protein interaction dependent heterodimerization. As a reporter system, these investigators fused HA-tagged dihydrofolate reductase (DHFR) in frame to the C_{ub} fragment. After reconstitution of functional ubiquitin, the DHFR is cleaved off, and this processing leads to an altered migration profile that can be observed *via* Western blotting using an antibody directed against the HA epitope tag [19].

This method was first explored in yeast using the homodimeric Gcn4 leucine zipper interaction, but was recently extended towards COS7 and HT1080 cells [20, 21]. In this last report, the investigators improved this approach by means of an elegant reporter system allowing easy forward and backward (disruption of interactions) screening. To this end the *E. coli* enzyme guanine phosphoribosyltransferase 2 (*gpt2*) was fused to the C_{ub} fragment. By adding an arginine residue after the cleavage site, the processed *gpt2* protein becomes a good substrate for the N-end rule pathway, resulting in rapid degradation. Using hypoxanthine-guanine-phosphoribosyltransferase-deficient HT1080 cells (HT1080HPRT⁻), selection for an interaction can be performed in 6-thioguanine(6TG)-containing medium, whilst counterselection occurs in hypoxanthine/aminopterin/thymine (HAT) medium (Figure 3a). The system was used to demonstrate the interactions between the TATA-binding protein 1 and nuclear factor kappaB, and the association between SP100B and heterochromatin protein1alpha [21].

As the ubiquitin system is functional in the nucleus as well as in the cytosol, this approach can be used to monitor interactions in both compartments. The ubiquitin-based split-protein assay can, in principle, be performed in every cell type, unless the *gpt2* reporter is used which restricts the system to HPRT-deficient cells.

Beta-galactosidase complementation

The use of beta-galactosidase complementation for detection of protein-protein interactions was first described by Rossi and coworkers. These investigators introduced mutations in discrete protein fragments of beta-galactosidase (DELTAalpha en DELTAoméga) to prevent spontaneous heterodimerization. Fusion of these fragments to the FKBP-rapamycin binding protein (FRAP) and FK506 binding protein 12 (FKBP12) allowed reconstitution of beta galactosidase activity in a rapamycin-dependent way [22]. A number of read-out systems to monitor activity can be applied, including the use of chemiluminescent substrates and flow cytometry after hypotonic loading of the cells with di-beta-galactopyranoside (Figure 3b). This beta-galactosidase complementation strategy was also successfully applied to study the kinetics of epidermal growth factor receptor dimerization in living cells [23].

An important advantage of the beta-galactosidase complementation strategy is the direct read-out associated with the analysed protein-interactions, thus allowing quantification of the interaction. Theoretically, the beta-galactosidase reconstitution should be highly sensitive as it is associated with enzymatic amplification (as opposed to DHFR complementation described below). This signal amplification also eliminates the need for overexpression, implying that the interactions can be studied under conditions that are physiologically more relevant. However, most substrates for beta-galactosidase require cell lysis, which may interfere with the interaction of interest and may thus lead to the loss of weak interactions, or to the detection of illegitimate interactions. Beta-galactosidase complementation is also cell-type and -compartment independent. A very interesting feature of this complementation strategy is its potential use in monitoring interactions *in vivo*. Expression of both bait and prey proteins can be obtained by creating knock-in mice for both. This may allow the study of protein interactions during development and in specific tissues [24]. FACS-based cell sorting after loading with di-beta-galactopyranoside may provide an extension towards cDNA library screening, but so far no novel interactions have been reported using this strategy.

Dihydrofolate reductase complementation

Dihydrofolate reductase (DHFR) complementation was originally described by Remy and Michnick [25]. The DHFR gene was dissected into two complementary fragments: F[1,2] and F[3]. Reconstitution of enzymatic activity was again realised by fusing interacting proteins to both fragments. Two read-outs can be

used: survival of DHFR negative cells in selective medium or binding of fluorescein-conjugated methotrexate as a quantitative assay based on fluorescence measurements (Figure 3c). The DHFR complementation strategy was successfully applied to the rapamycin-dependent binding of FKBP to FRAP using both read-out systems [25]. This strategy was also used in the analysis of erythropoietin receptor dimerization [26]. In these experiments the intracellular domain of this type I cytokine receptor was exchanged for the DHFR fragments. Using variable length linkers between the transmembrane domain and the DHFR fragments, these investigators could demonstrate the existence of a preformed homo-dimeric receptor complex, while binding of agonists led to an altered conformation, resulting in closer proximity of the transmembrane domains. Again, this complementation strategy allows for quantification of the interaction, although the system is less sensitive than beta-galactosidase complementation as no enzymatic amplification is obtained. Application of this approach is cell-type and -compartment independent, and, in principle, allows detection of modification-dependent interactions. DHFR complementation can be used for screening complex cDNA libraries in DHFR-deficient cells.

Béta-lactamase complementation

In this recently developed complementation assay, two proteins are fused to fragments of beta-lactamase (alpha197 and oméga198; Figure 3d) [27]. Advantages of using beta-lactamase include the small size of the fragments (< 19 kDa), the absence of endogenous lactamase activity in mammalian cells, and the enzymatic amplification associated with the interaction resulting in a sensitive system. The availability of a cell-permeable fluorescent substrate (CCF2/AM) permits analysis in living cells. The lactamase chimeras were optimised by insertion of a long 3(Gly₄Ser) linker, and by screening the effect of random tripeptides in the joining region. The latter was assayed in *E. coli* cells via the interaction between c-Fos and c-Jun. This resulted in a strong improvement of interaction-dependent beta-lactamase activity, an effect that is also observed for other interactions. Using these optimised fragments, the method allowed detection of the rapamycin-dependent interaction between FKBP12 and the FKBP-rapamycin binding domain of FRAP (FRB) in mammalian cells. When the FKBP12-oméga198 chimeric construct was anchored to the membrane by fusion with the extracellular and transmembrane part of the epidermal growth factor receptor, similar beta-lactamase activities were observed after complementation with the cytoplasmic FRB-alpha197 protein. This suggests

that the strategy can be used to study membrane-proximal events such as signal transduction processes. Galarneau and colleagues also published the development of a protein complementation assay based on beta-lactamase. As substrates they use both nitrocefin, a colorimetric substrate that requires cell lysis and the CCF2/AM cell-permeable substrate. The method was applied to a number of model interactions including the homodimerization of Smad3 and the interaction between protein kinase B (PKB) and its substrate Bad [28]. Because of the small size of the fragments and the flexibility introduced by using long linker sequences, this complementation system may offer a broader application range, and may be better suited for cDNA library screening.

FRET/BRET - BASED STRATEGIES

Fluorescence technology and image analysis software have been recently developed and optimised resulting in a myriad of applications and strategies. Fluorescence in protein interaction research was initially used in co-localization studies with superimposed images of two (or more) fluorescent labels. Although results obtained using this technique are indicative of protein interactions, direct interaction cannot be formally shown. A strategy to address this problem is based on energy transfer between two different fluorophores. Excitation of one fluorophore (donor) results in light emission that excites another proximal fluorophore (acceptor), ultimately leading to emission at a different wavelength from the acceptor fluorophore as if it had been excited directly. Fluorescent labelling of purified proteins followed by micro-injection in living cells allowed the detection of protein-protein interactions *via* fluorescence resonance energy transfer [29]. The strategy was very labour intensive and technically demanding, which has prevented its widespread use. Introduction of green fluorescent protein (GFP) variants has allowed simple transfection of bait and prey chimeras in cells, and omits the need for extensive cell manipulation (*Figure 4a*). Successful applications include the phosphorylation-dependent interaction between the epidermal growth factor receptor (EGFR) and the growth receptor-bound protein 2 (grb-2) [30], the microaggregation of the gonadotrophin-releasing hormone receptor [31] and the dimerization of receptor protein-tyrosine phosphatase alpha [32]. GFP-based FRET also allows detection of protein interactions in specialized cell compartments and has been used to demonstrate the interaction between the transcription factors Pit-1 and c-Ets-1 in the nucleus [33], and between Bcl-2 and Bax in mitochondria [34]. Fluorescent labeled antibodies and ligands have been successfully used in

FRET-based studies of receptor-ligand [35] and extracellular receptor-receptor interactions [36].

In bioluminescence resonance energy transfer (BRET), excitation of the acceptor fluorophore is achieved *via* enzymatic bioluminescence instead of a donor fluorophore. In typical BRET experiments in mammalian cells, Renilla luciferase is fused to one protein while enhanced yellow fluorescent protein is fused to the other protein. BRET activity can be observed by adding the cell permeable luciferase substrate coelenterazine to the cells (Figure 4b). BRET has been successfully applied in the study of dimerization of G-protein coupled receptors and the interaction between these receptor and beta-arrestin [37, 38].

An important advantage for FRET/BRET is the possibility to monitor interaction in time, thus allowing the research of dynamic processes. Both FRET and BRET can theoretically be used in all cell types and compartments. Proteins should be within a proximity of 10-100 angstrom for measurements with both FRET and BRET. Disadvantages include the requirement for proper orientation of the donor and acceptor dipoles without the interference of the interacting proteins. The techniques may require optimisation of the chimeric constructs which suggests a high number of false negatives in screening experiments. BRET has potential advantages over FRET because it does not require excitation of the donor molecule. Problems with photobleaching and autofluorescence are thus limited in BRET.

A CYTOKINE-RECEPTOR-BASED INTERACTION TRAP: MAPPIT

The mammalian protein-protein interaction trap (MAPPIT) is based on insights obtained from type I cytokine-receptor activation and signalling. Accumulating evidence suggests that these preformed receptors undergo conformational changes after ligand binding, leading to cross-phosphorylation and activation of associated JAK kinases. These JAKs then phosphorylate tyrosines within the cytoplasmic tails of the receptors, which is followed by recruitment of signal transduction proteins to the activated receptor complex. Important signalling components that dock on the activated receptor, include the signal transducer and activator of transcription (STAT) proteins, which are latent transcription factors that migrate to the nucleus after activation and dimerization. In the MAPPIT system, the bait protein is fused to a signalling-deficient receptor, *e.g.* a

receptor lacking functional tyrosines, while the prey protein is fused to a receptor fragment containing STAT recruitment sites. An interaction thus results in STAT activation and can be monitored by a STAT-responsive reporter (Figure 5). The system takes full advantage of the shuttling of STAT proteins between cytoplasm and nucleus, as this implies that effector and interactor zones are separated. This may result in lower incidence of false positives. The MAPPIT system was validated in HEK293T cells *via* the p53-SV40 LargeT (SVT) interaction and the phosphorylation-dependent interaction between the erythropoietin receptor (EpoR) Y402 motif and the cytokine inducible SH2-containing protein (CIS).

MAPPIT was extended to cDNA library screening by generating a stable cell line with three distinct properties. This HEK293-16 cell clone allows rapid integration of bait chimeras *via* targeted recombination, infection with murine ecotropic retrovirus for prey integration, and selection *via* a stably integrated puromycin resistance cassette fused to the STAT-responsive reporter. Screening of the EpoR Y402 motif with a retroviral HEK293 cDNA library resulted in the identification of CIS2, a homologue of CIS [39].

Conceptually, MAPPIT is more flexible when orientation requirements of the prey construct are considered. Indeed, although the underlying mechanism is not really understood, the JAKs appear to be very flexible in their choice of substrates. Many chimeric receptors with artificial intracellular regions that can be derived from other receptors, still allow a significant STAT response (*e.g.* [40]). Due to its design, MAPPIT detects interactions in the cytoplasm near the cell membrane and is therefore ideally suited to study signalling pathways.

Background signals appear to be limited, as long as the bait protein itself does not lead to reporter introduction. This problem can be circumvented by the use of other prey/STAT/reporter combinations. Since MAPPIT is an inducible system, an additional control over background signals is obtained. Stimulus-independent signals caused by specific prey constructs can thus be easily identified.

It is well known that the extracellular parts of some type I cytokine receptors can be swapped, thus allowing activation of the intracellular domain by addition of the ligand that corresponds to the other receptor. As such, cell clones obtained by screening can be transfected with a construct encoding a mutated bait,

fused to such a swapped chimeric receptor, followed by stimulation with the cognate ligand. Hence, this permits an immediate preliminary structure-function analysis of the bait-prey interaction. Moreover, transfection of a chimeric receptor lacking the bait may distinguish between interactions that occur on the receptor backbone instead of the bait itself.

INTERACTION-DEPENDENT PROTEIN SPLICING

The discovery of protein splicing, *i.e.* the posttranslational excision of a protein fragment, led to the development of a number of novel applications, including advanced protein engineering (reviewed in [41]). Ozawa and co-workers successfully used protein splicing to detect protein interactions. In this strategy, a tripartite bait construction is used, which consists of a reporter fragment fused to a forward splicing site (dnaEn) and the bait protein. The prey construct similarly consists of the complementary fragment of the reporter, the reverse protein splice site (dnaEc) and the prey protein. Interaction results in excision of the bait-prey complex *via* the splice sites and concomitant ligation of the reporter fragments, leading to reconstitution of enzymatic or fluorescent properties (*Figure 6*). The technology was first demonstrated in bacteria using a GFP reporter [42], and was very recently extended to Chinese hamster ovary (CHO) cells using luciferase fragments for detection. These investigators were able to demonstrate the tyrosine phosphorylation-dependent interaction between a peptide fragment of insulin receptor substrate 1 (IRS-1) containing the Y941 motif, and the amino-terminal SH2 domain of phosphatidyl inositol 3-kinase (PI3K) [43].

Protein splicing is a self-catalysing event which does not need the activity of other enzymes. Reconstitution of GFP can thus be monitored in all cell lines and cell compartments and may directly reflect kinetic properties of the interaction. This strategy could also be theoretically applied to the detection of interactions *in vivo* after a knock-in of both the bait and the prey. The screening of cDNA libraries should be possible but has not been demonstrated yet. The same drawbacks as found for protein complementation strategies also apply to protein splicing. Large bait or prey proteins, or orientation of the complex may interfere with the splicing event and thus result in the occurrence of false negative interactions. Optimisation of the bait and prey constructs may therefore be required to detect the interaction.

CONCLUSION

An important strategy in the functional annotation of a gene product is the positioning of the protein in a pathway or subcellular complex. This can be achieved by screening for protein partners, *e.g.* looking at the interaction profile. The yeast two-hybrid technique is herein the method of choice due to its robustness and low operation cost, and is currently being evaluated for the construction of "complete" interaction maps (reviewed in [44, 45]). Yet, systematic identification of protein complexes from yeast cells *via* highly efficient purification tags followed by mass spectrometry showed higher detection efficiency when compared to yeast two-hybrid mapping [3, 4]. Although both techniques are clearly useful in the creation of rough interaction maps, they still suffer from intrinsic limitations. Both procedures have a high incidence of false positives, and probably also of false negatives. Mammalian systems have great potential to circumvent some of the problems. However, most of these systems have been developed rather recently and have not yet found widespread application, which may also be due to their complexity and technological requirements. Moreover, screening of complex cDNA libraries remains difficult and time-consuming. Reasons for this are the requirement for stable expression of both bait and prey in lengthy selection strategies, although recent solutions have been provided by retroviral strategies or by targeted recombination. Retroviral systems may also solve the problems associated with bulk cDNA transfection procedures, which are not only inefficient and labour-intensive, but also require sensitive assays to compensate for a signal dilution effect [46, 47]. An alternative may be the amplification or extrachromosomal maintenance of plasmids *via* respectively SV40 LargeT or EBNA-1 [14, 46]. Selection strategies are dependent on the system used. In the reporter-based strategies such as the mammalian two-hybrid system and MAPPIT, the promoter used for the readout can be fused to a selector gene such as a hygromycin or puromycin resistance cassette [13, 39], or membrane markers allowing the use of fluorescence- (FACS) or magnetobead-assisted cell sorting (MACS) [13]. Other mammalian interaction detection strategies rely on inherent properties that can be directly monitored such as FRET or DHFR complementation. The issue of random cDNA library screening may become less important since more academic and particularly commercial effort is being put into the development of ORFeomes and cDNA collections. Together with advances in robotics and signal detection machines, systematic testing of every potential interaction between cDNAs in an arrayed fashion will become feasible [15].

A universal method for studying protein interactions has not yet been developed and may well prove to be utopian given the complexity underlying protein interactions. Hence, it may be necessary to employ "tailor-made systems" depending on the questions that need to be addressed. Variables that influence the choice of a particular system include (i) the involvement of modifications or conformational requirements in the interaction, (ii) the subcellular compartment where the interaction occurs, (iii) quantification of the interaction and (iv) interaction dynamics. Other issues involve application of the system in the creation of interaction maps and the screening of complex cDNA libraries, or relate to the technological requirements needed to use a particular system. All of the mammalian systems available to date have specific properties and requirements, allowing a well-considered choice for the biological question(s) that needs to be addressed.

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References

1. Dyson N. 1998. The regulation of E2F by pRB-family proteins. *Genes Dev.* 12: 2245.
2. Hazbun T R, Fields S. 2001. Networking proteins in yeast. *Proc. Natl. Acad. Sci. USA* 98: 4277.
3. Ho Y, Gruhler A, Heilbut A, Bader G D, Moore L, Adams S L, Millar A, Taylor P, Bennett K, Boutilier K, *et al.* 2002. Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. *Nature* 415: 180.
4. Gavin A C, Bosche M, Krause R, Grandi P, Marzioch M, Bauer A, Schultz J, Rick J M, Michon A M, Cruciat C M, *et al.* 2002. Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature* 415: 141.

5. Fields S, Song O. 1989. A novel genetic system to detect protein-protein interactions. *Nature* 340: 245.
6. Iwabuchi K, Li B, Bartel P, Fields S. 1993. Use of the two-hybrid system to identify the domain of p53 involved in oligomerization. *Oncogene* 8: 1693.
7. Uetz P, Giot L, Cagney G, Mansfield T A, Judson R S, Knight J R, Lockshon D, Narayan V, Srinivasan M, Pochart P, *et al.* 2000. A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. *Nature* 403: 623.
8. Ito T, Tashiro K, Muta S, Ozawa R, Chiba T, Nishizawa M, Yamamoto K, Kuhara S, Sakaki Y. 2000. Toward a protein-protein interaction map of the budding yeast: A comprehensive system to examine two-hybrid interactions in all possible combinations between the yeast proteins. *Proc. Natl. Acad. Sci. USA* 97: 1143.
9. Schwikowski B, Uetz P, Fields S. 2000. A network of protein-protein interactions in yeast. *Nat. Biotechnol.* 18: 1257.
10. Vidal M, Brachmann R K, Fattaey A, Harlow E, Boeke J D. 1996. Reverse two-hybrid and one-hybrid systems to detect dissociation of protein-protein and DNA-protein interactions. *Proc. Natl. Acad. Sci. USA* 93: 10315.
11. Park S H, Raines R T. 2000. Genetic selection for dissociative inhibitors of designated protein-protein interactions. *Nat. Biotechnol.* 18: 847.
12. Dang C V, Barrett J, Villa-Garcia M, Resar L M, Kato G J, Fearon E R. 1991. Intracellular leucine zipper interactions suggest c-Myc hetero-oligomerization. *Mol. Cell. Biol.* 11: 954.
13. Fearon E R, Finkel T, Gillison M L, Kennedy S P, Casella J F, Tomaselli G F, Morrow J S, Van Dang C. 1992. Karyoplasmic interaction selection strategy: a general strategy to detect protein-protein interactions in mammalian cells. *Proc. Natl. Acad. Sci. USA* 89: 7958.
14. Shioda T, Andriole S, Yahata T, Isselbacher K J. 2000. A green fluorescent protein-reporter mammalian two-hybrid system with extrachromosomal maintenance of a prey expression plasmid: application to interaction screening. *Proc. Natl. Acad. Sci. USA* 97: 5220.

15. Suzuki H, Fukunishi Y, Kagawa I, Saito R, Oda H, Endo T, Kondo S, Bono H, Okazaki Y, Hayashizaki Y. 2001. Protein-protein interaction panel using mouse full-length cDNAs. *Genome Res.* 11: 1758.
16. Aronheim A, Zandi E, Hennemann H, Elledge S J, Karin M. 1997. Isolation of an AP-1 repressor by a novel method for detecting protein- protein interactions. *Mol. Cell Biol.* 17: 3094.
17. Broder Y C, Katz S, Aronheim A. 1998. The ras recruitment system, a novel approach to the study of protein- protein interactions. *Curr. Biol.* 8: 1121.
18. Maroun M, Aronheim A. 1999. A novel *in vivo* assay for the analysis of protein-protein interaction. *Nucleic Acids Res.* 27: e4.
19. Johnsson N, Varshavsky A. 1994. Split ubiquitin as a sensor of protein interactions *in vivo*. *Proc. Natl. Acad. Sci. USA* 91: 10340.
20. Pelletier J N, Arndt K M, Pluckthun A, Michnick S W. 1999. An *in vivo* library-versus-library selection of optimized protein-protein interactions. *Nat. Biotechnol.* 17: 683.
21. Rojo-Niersbach E, Morley D, Heck S, Lehming N. 2000. A new method for the selection of protein interactions in mammalian cells. *Biochem. J.* 348 Pt 3: 585.
22. Rossi F, Charlton C A, Blau H M. 1997. Monitoring protein-protein interactions in intact eukaryotic cells by beta-galactosidase complementation. *Proc. Natl. Acad. Sci. USA* 94: 8405.
23. Blakely B T, Rossi F M, Tillotson B, Palmer M, Estelles A, Blau H M. 2000. Epidermal growth factor receptor dimerization monitored in live cells. *Nat. Biotechnol.* 18: 218.
24. Rossi F M, Blakely B T, Blau H M. 2000. Interaction blues: protein interactions monitored in live mammalian cells by beta-galactosidase complementation. (Review) *Trends Cell Biol.* 10: 119.

25. Remy I, Michnick S W. 1999. Clonal selection and *in vivo* quantitation of protein interactions with protein-fragment complementation assays. *Proc. Natl. Acad. Sci. USA* 96: 5394.
26. Remy I, Wilson I A, Michnick S W. 1999. Erythropoietin receptor activation by a ligand-induced conformation change. *Science* 283: 990.
27. Wehrman T, Kleaveland B, Her J H, Balint R F, Blau H M. 2002. Protein-protein interactions monitored in mammalian cells *via* complementation of beta-lactamase enzyme fragments. *Proc. Natl. Acad. Sci. USA* 99: 3469.
28. Galarneau A, Primeau M, Trudeau L E, Michnick S W. 2002. beta-lactamase protein fragment complementation assays as *in vivo* and *in vitro* sensors of protein interactions. *Nat Biotechnol.* 20: 619.
29. Adams S R, Harootunian A T, Buechler Y J, Taylor S S, Tsien R Y. 1991. Fluorescence ratio imaging of cyclic AMP in single cells. *Nature* 349: 694.
30. Sorkin A, McClure M, Huang F, Carter R. 2000. Interaction of EGF receptor and grb2 in living cells visualized by fluorescence resonance energy transfer (FRET) microscopy. *Curr. Biol.* 10: 1395.
31. Cornea A, Janovick J A, Maya-Nunez G, Conn P M. 2001. Gonadotropin-releasing hormone receptor microaggregation. Rate monitored by fluorescence resonance energy transfer. *J. Biol. Chem.* 276: 2153.
32. Tertoolen L G, Blanchetot C, Jiang G, Overvoorde J, Gadella T W Jr, Hunter T, Hertog J J. 2001. Dimerization of Receptor Protein-Tyrosine Phosphatase alpha in living cells. *BMC. Cell Biol.* 2: 8.
33. Day R N. 1998. Visualization of Pit-1 transcription factor interactions in the living cell nucleus by fluorescence resonance energy transfer microscopy. *Mol. Endocrinol.* 12: 1410.
34. Mahajan N P, Linder K, Berry G, Gordon G W, Heim R, Herman B. 1998. Bcl-2 and Bax interactions in mitochondria probed with green fluorescent protein and fluorescence resonance energy transfer. *Nat. Biotechnol.* 16: 547.

35. Gadella T W Jr, Jovin T M. 1995. Oligomerization of epidermal growth factor receptors on A431 cells studied by time-resolved fluorescence imaging microscopy. A stereochemical model for tyrosine kinase receptor activation. *J. Cell Biol.* 129: 1543.
36. Damjanovich S, Bene L, Matko J, Alileche A, Goldman C K, Sharrow S, Waldmann T A. 1997. Preassembly of interleukin 2 (IL-2) receptor subunits on resting Kit 225 K6 T cells and their modulation by IL-2, IL-7, and IL-15: a fluorescence resonance energy transfer study. *Proc. Natl. Acad. Sci. USA* 94: 13134.
37. Angers S, Salahpour A, Joly E, Hilalret S, Chelsky D, Dennis M, Bouvier M. 2000. Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). *Proc. Natl. Acad. Sci. USA* 97: 3684.
38. Kroeger K M, Hanyaloglu A C, Seeber R M, Miles L E, Eidne K A. 2001. Constitutive and agonist-dependent homo-oligomerization of the thyrotropin-releasing hormone receptor. Detection in living cells using bioluminescence resonance energy transfer. *J. Biol. Chem.* 276: 12736.
39. Eyckerman S, Verhee A, der Heyden J V, Lemmens I, Ostade X V, Vandekerckhove J, Tavernier J. 2001. Design and application of a cytokine-receptor-based interaction trap. *Nat. Cell Biol.* 3: 1114.
40. Behrmann I, Janzen C, Gerhartz C, Schmitz-Van de Leur H, Hermanns H, Heesel B, Graeve L, Horn F, Tavernier J, Heinrich P C. 1997. A single STAT recruitment module in a chimeric cytokine receptor complex is sufficient for STAT activation. *J. Biol. Chem.* 272: 5269.
41. Paulus H. 2000. Protein splicing and related forms of protein autoprocessing. (Review) *Annu. Rev. Biochem.* 69: 447.
42. Ozawa T, Nogami S, Sato M, Ohya Y, Umezawa Y. 2000. A fluorescent indicator for detecting protein-protein interactions *in vivo* based on protein splicing. *Anal. Chem.* 72: 5151.

43. Ozawa T, Kaihara A, Sato M, Tachihara K, Umezawa Y. 2001. Split luciferase as an optical probe for detecting protein-protein interactions in mammalian cells based on protein splicing. *Anal. Chem.* 73: 2516.
44. Walhout A J, Vidal M. 2001. Protein interaction maps for model organisms. (Review) *Nat. Rev. Mol. Cell Biol.* 2: 55.
45. Tucker C L, Gera J F, Uetz P. 2001. Towards an understanding of complex protein networks. (Review) *Trends Cell Biol.* 11: 102.
46. Vasavada H A, Ganguly S, Germino F J, Wang Z X, Weissman S M. 1991. A contingent replication assay for the detection of protein-protein interactions in animal cells. *Proc. Natl. Acad. Sci. USA* 88: 10686.
47. Sotiropoulos A, Gineitis D, Copeland J, Treisman R. 1999. Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. *Cell* 98: 159.

Figures

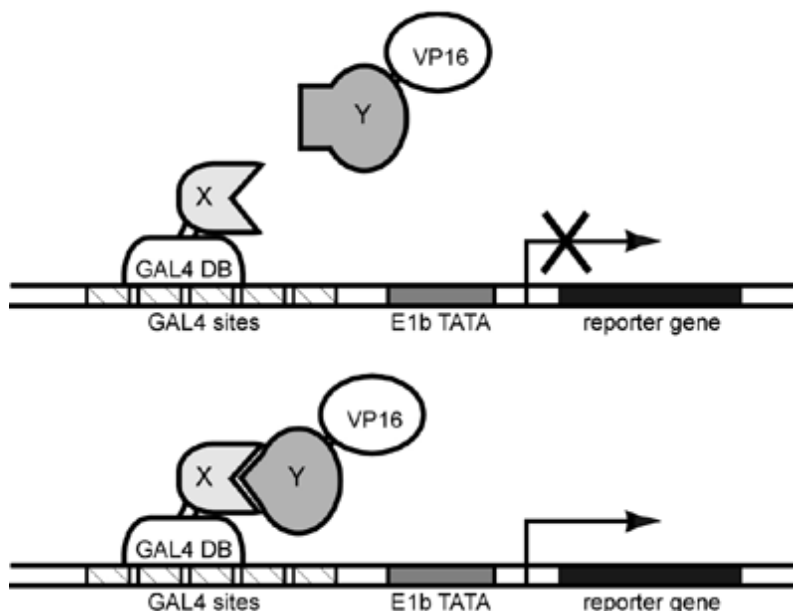


Figure 1

Layout of the mammalian two-hybrid method.

Interaction between two proteins leads to reconstitution of a functional transcription factor, a process that can be monitored by use of reporter genes (DB: DNA binding domain).

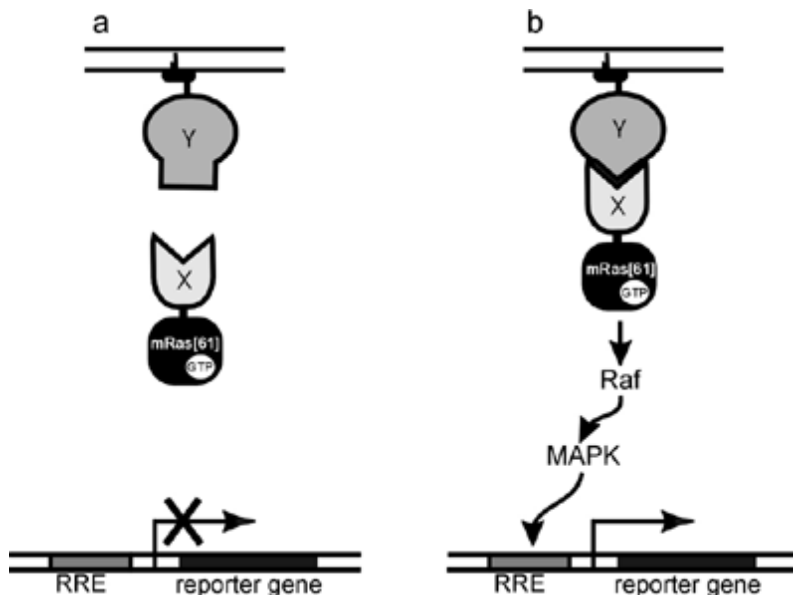


Figure 2

Schematic representation of the mammalian Ras recruitment system (mRRS).

The bait protein is fused to mutated Ras that cannot bind to the membrane, while the prey protein contains a membrane localisation signal. Interaction leads to recruitment of the chimeric Ras protein to the membrane which is sufficient to activate the Raf/Mitogen activated protein kinase (MAPK) pathway, ultimately resulting in induction of a reporter gene containing Ras responsive elements (RRE).

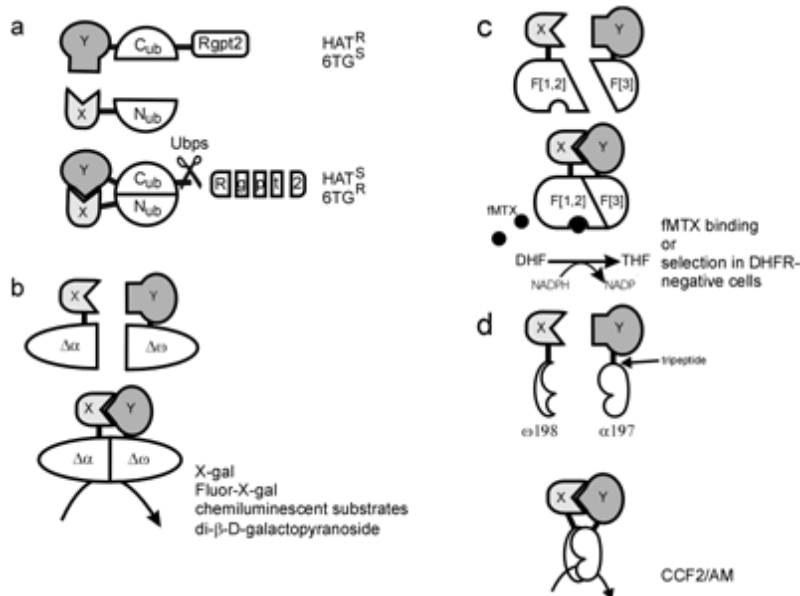


Figure 3

Overview of the different protein complementation assays.

- a.** Reconstitution of functional ubiquitin *via* a protein-protein interaction, results in cleavage of gpt2 by the ubiquitin-specific proteases (Ubps). The cleaved protein starts with an arginine residue, and is therefore rapidly degraded by the N-end rule pathway, resulting in resistance *versus* 6-thioguanine (6TG) and sensitivity for hypoxanthine/aminopterin/thymine (HAT).
- b.** Complementation of two beta-galactosidase fragments (deltaalpha en deltaomega) results in the reconstitution of enzymatic activity which can be detected by use of various substrates.
- c.** Reconstitution of DHFR activity is monitored *via* labelled methotrexate (fMTX) or by survival of DHFR deficient cells (DHF: dihydrofolate, THF: tetrahydrofolate).
- d.** Reconstitution of beta-lactamase activity by interaction-dependent dimerization of the alpha197 and

oméga198 fragments. A specific tripeptide sequence in the alpha197 chimeric protein ameliorates the functionality of the enzyme after complementation.

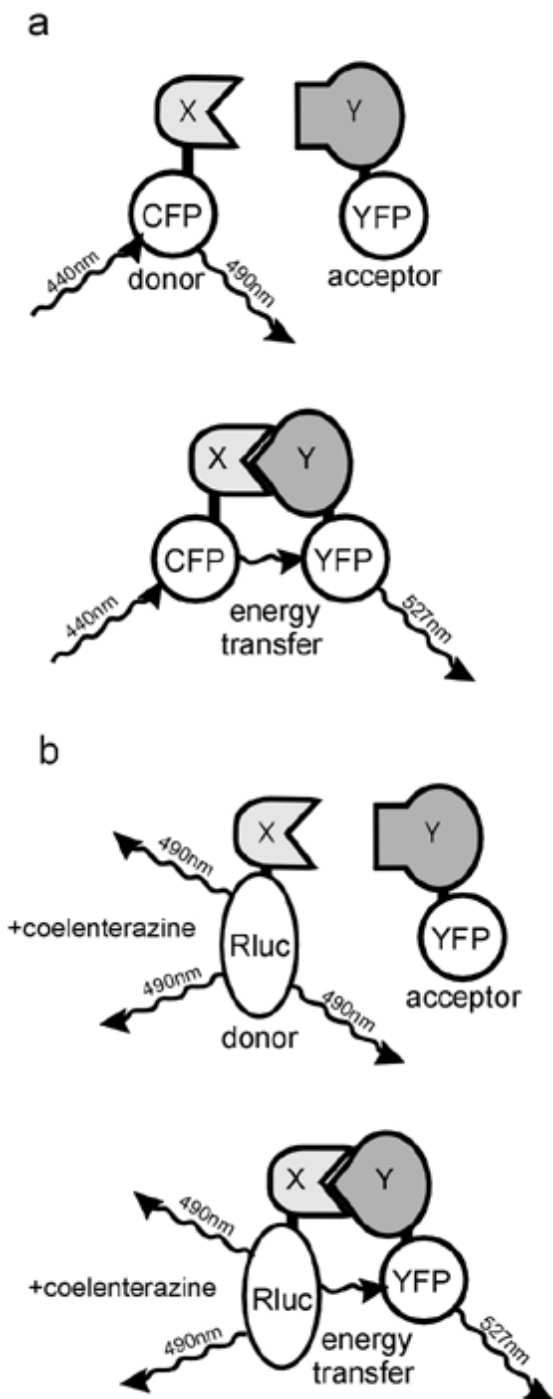


Figure 4

Energy transfer in the detection of protein-protein interactions.

a. Fluorescence resonance energy transfer (FRET):

Excitation of the donor fluorophore (cyan fluorescent protein, CFP) leads to light emission that can excite the acceptor fluorophore (yellow fluorescent protein, YFP) if these proteins are in close proximity (10-100 angstrom). This ultimately results in emission at a different wavelength which can be monitored.

b. Bioluminescence resonance energy transfer (BRET):

Excitation of a proximal (10-100 angstrom) fluorophore is obtained *via* a chemiluminescent reaction catalysed by Renilla luciferase (Rluc) in the presence of coelenterazine.

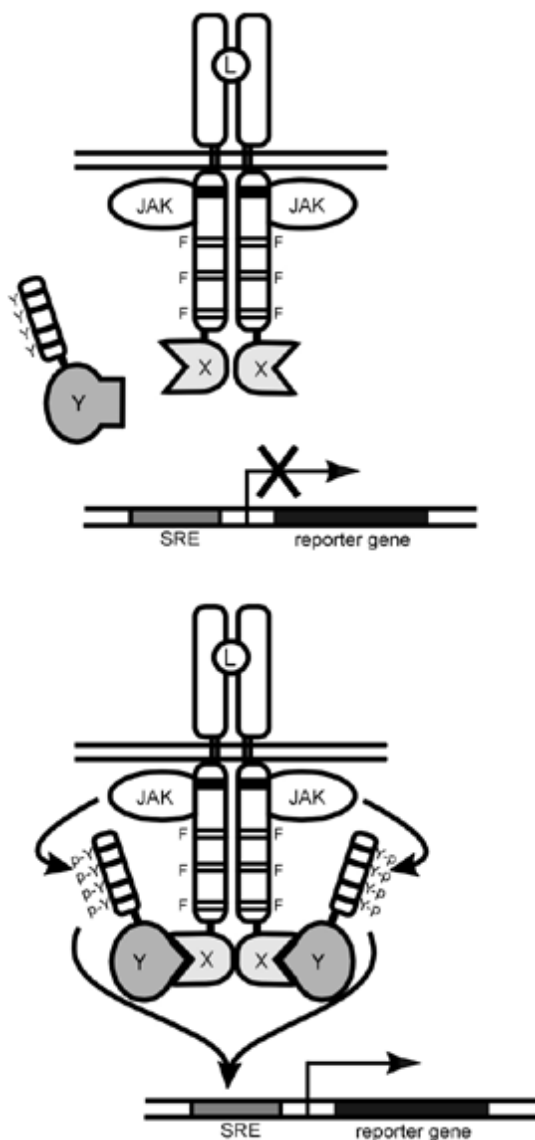
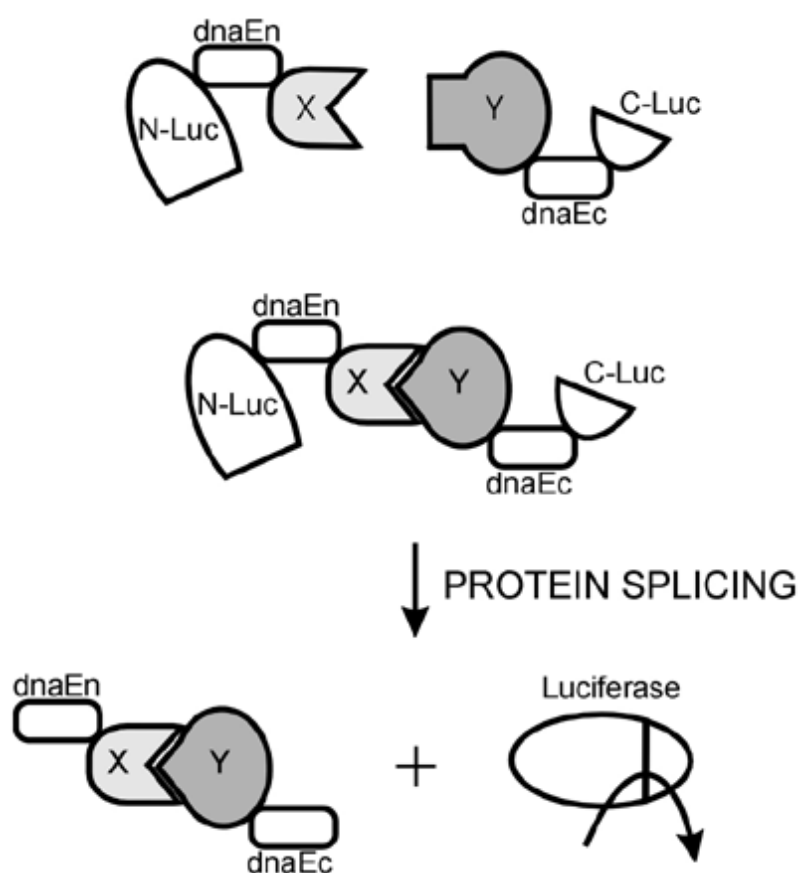


Figure 5**Principle of the mammalian protein-protein interaction map (MAPPIT).**

Binding of the ligand to a signalling-deficient receptor can still lead to activation of the associated JAK kinases, but does not result in detectable gene induction. When a receptor fragment containing functional STAT docking sites, is recruited to the activated complex *via* a bait-prey interaction, this leads to activation of STATs and induction of (reporter) genes containing STAT responsive elements (SRE).

**Figure 6**

Principle of interaction-dependent protein splicing.

Interaction between bait and prey results in excision of dnaEn-bait and dnaEc-prey proteins when these are in close proximity. This event is followed by protein ligation of the reporter fragments (N-luc and C-luc) resulting in a functional reporter protein (luciferase).

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In this issue

○ Methods to map protein interactions in mammalian cells: different tools to address different questions

Sven Eyckerman, Jan Tavernier

two-hybrid, enzymatic complementation, FRET/BRET, MAPPIT, protein splicing.

○ Absence of endogenous interleukin-10 enhances the evolution of acute lung injury

Salvatore Cuzzocrea, Emanuela Mazzon, Laura Dugo, Ivana Serraino, Rosanna Di Paola, Tiziana Genovese, Angela De Sarro, Achille P. Caputi
carrageenan, inflammation, nitric oxide, peroxynitrite, superoxide dismutase, IL-10.

○ Dose-effect of interleukin-10 and its immunoregulatory role in Crohn's disease

Alain Schmit, Monica Carol, Fabienne Robert, Patrick Bontems, Jean-Jacques Houben, André Van Gossum, Michel Goldman, Françoise Mascart
Crohn's disease, interleukin-10, tumor necrosis factor- α .

○ Expression of genes for cytokines and cytokine-related functions in leukocytes infected with Herpes simplex virus: comparison between resistant and susceptible mouse strains

Søren R. Paludan, Jesper Melchjorsen, Lene Malmgaard, Søren C. Mogensen
leukocytes, cytokines, cDNA arrays, Herpes simplex virus.

○ Mycophenolic acid and methotrexate inhibit lymphocyte cytokine production via different mechanisms

Sacha de Lathouder, Andreas H. Gerards, Els R. de Groot, Marijke Valkhof, Lucien A. Aarden
lymphocytes, apoptosis, whole blood, activation markers, drug effects, immunosuppression.

○ Cytokine pattern in cystic fibrosis patients during antibiotic therapy and gene therapy using adenoviral vector

Philippe Reix, Gabriel Bellon, Jacques Bienvenu, Andrea Pavirani, Hélène Levrey-Hadden
cytokine, cystic fibrosis, adenoviral vector, antibiotic.

○ Induction of interleukin-2 receptor alpha (IL-2Ralpha) expression by interleukin-2: important role of the interleukin-2 receptor beta chain region between the two Stat5 docking sites

Véronique Imbert, Roger Rezzonico, Patrick Reichenbach, Markus Nabholz
Stat5, IL-2 receptor, T lymphocytes, CD25.

○ Bronchopulmonary hyperreactivity and lung eosinophil sequestration but not their migration to the alveolar compartment are independent of interleukin-5 in allergic mice

Barbara Proust, Claude Ruffié, Jean Lefort, B. Boris Vargaftig
bronchopulmonary hyperreactivity, eosinophilia, IL-5, TRFK-5.

○ Complex network of cytokines activating murine microglial cell activity against *Acanthamoeba castellanii*

Nunzia Benedetto, Claude Auriault
A. castellanii, cytokine, MGC.

○ The mechanism by which interleukin-1 beta reduces net fluid absorption from the rat colon

Sawsan Ibrahim Kreydiyyeh, Rana Al-Sadi

Interleukin-1 beta, Na⁺K⁺ ATPase, NaKCl2, Cl⁻ secretion, Na⁺ absorption, colon.

○ Proinflammatory interferon-gamma - inducing monokines (interleukin-12, interleukin-18, interleukin-15) - serum profile in patients with systemic lupus erythematosus

Ewa Robak, Tadeusz Robak, Anna Wozniacka, Malgorzata Zak-Prelich, Anna Sysa-Jedrzejowska, Henryk Stepien

SLE, Interferon gamma, IL-12, IL-15, IL-18, disease activity.

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