Screening for extracellular protein – protein interactions in a novel yeast growth selection system

INAUGURALDISSERTATION

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

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Basel 2004
Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag von
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Basel, den 6. April 2004

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1. SUMMARY

The recent sequencing of entire eukaryotic genomes revealed a great number of open reading frames (ORFs), most of which potentially code for proteins of unknown function. Identifying interaction partners may facilitate the functional characterisation of unknown gene products. Moreover, large-scale approaches to identify protein interactions may be used to untangle the complete interaction network of all the gene products expressed in a given organism, the so-called interactome. Currently, the yeast two-hybrid system is the most widely used genetic assay for large-scale detection of protein–protein interactions and it has successfully been applied to map the interactome of several organisms. However, since interactions in this system are detected in the nuclear environment, it does not account for the particular biochemical requirements of extracellular and integral membrane proteins.

In the first part of this thesis, I describe a novel genetic growth selection system to detect interactions between extracellular and transmembrane proteins in a topologically extracellular compartment of *Saccharomyces cerevisiae*. In this system the proteins of interest are expressed as fusions to mutually complementing mutant derivatives of the yeast ER resident transmembrane receptor Ire1p. Interaction between the proteins of interest causes dimerisation of the Ire1p moieties allowing for complementation and activation of Ire1p. Active Ire1p in turn triggers a signalling cascade, which induces expression of selectable reporter genes in the nucleus and promotes growth under selective conditions. The feasibility of this system to monitor interactions between extracellular proteins was demonstrated by specific pairings of epitope and single-chain Fv (scFv) antibody fragments.

In part two, I describe the application of this system in a screening procedure to select scFv antibody fragments that specifically bind to human Interleukin-13 (hIL-13). In a first round, hIL-13 binders were selected from a scFv-antibody library by yeast growth under selective conditions. In order to improve their binding affinity, parts of the scFv fragments obtained from the primary screening were randomised by homologous recombination in yeast and subjected to growth selection under increased selective conditions.
1. ZUSAMMENFASSUNG


Diese Methode wurde in der Folge angewandt, um in einer ersten Selektionsrunde aus einem Pool von scFv Fragmenten diejenigen zu selektionieren, die spezifisch an humanes Interleukin-13 (hIL-13) binden. Um die Bindungsaffinität dieser primären Binder zu verbessern wurden sie, durch homologe Rekombination in Hefezellen randomisiert und einer zweiten strengeren Wachstumsselektion unterworfen.
2. INTRODUCTION

Vital processes such as adaptation to a given environment, reproduction and differentiation are controlled via communication between cells and cell compartments. Emitted communication signals (e.g. hormones) or environmental conditions (e.g. extracellular matrix composition) are in many cases sensed by a variety of specific cell surface receptors and transduced to the nucleus via signalling cascades that eventually activate specific subsets of genes in the nucleus. In order to integrate a multitude of concomitant signals into one expressional output, many signalling pathways cross talk with each other to build a complex signalling network. These signalling processes are controlled by protein – protein interactions, which are further regulated by protein modifications. Knowing interaction partners of a given gene product would thus facilitate the characterisation of gene functions in living organisms.

The recent sequencing of entire eukaryotic genomes revealed potential open reading frames (ORFs) and provided basic information for further characterization of all the gene products and their interaction partners expressed in a given organism or a cell type. A number of techniques have been described which allow studying protein interaction networks in large-scale procedures. These techniques may be grouped into two categories: i) biochemical approaches in which interactions between proteins are detected or analysed in vitro, for example cross-linking and co-purification (phage-display or ribosomal-display also belong to this category since selection steps in this methods also occur in vitro), and ii) genetic approaches, of which the classical yeast-two-hybrid is the most popular method to detect protein – protein interactions in vivo. Both biochemical and genetic approaches have important advantages as well as limitations and all of them are rather complimentary than substitutes for each other.

2. 1. Biochemical approaches

Conventional biochemical approaches such as cross-linking, co-immunoprecipitation and co-fractionation by chromatography have been used in the past to detect protein – protein interactions. One disadvantage of purely biochemical methods is that
interacting proteins often exist in low abundance in living cells and are therefore difficult to detect. In contrast, technologies such as phage-display, yeast-surface-display and ribosomal-display allow controlled expression of the proteins of interest (POI) and have in common that very high numbers of clones, each expressing a distinct protein, can be screened in a short time for the ability of the POI to bind a certain target. As a rough comparison, billions of clones can be screened within one week with phage-display while only millions of clones can be screened in two to four weeks with yeast-two-hybrids. Since in surface-display as well as in ribosomal-display selection of clones through binding to a target protein occurs in vitro, binding conditions can be modified and thus adjusted to the requirements of every given target protein. As an alternative to these in vitro screening methods, protein complexes formed in vivo can be purified from cell lysates in large scale. Subsequent determination of the complex members by mass spectrometry has been shown to be a very powerful tool to analyse the interactome of yeast. An important limitation of biochemical assays, however, is that target proteins or studied protein complexes must be purified. Depending on the features they bear, many proteins can be extremely difficult to purify. Multi-spanning transmembrane proteins, for example, contain up to seven hydrophobic transmembrane domains, which complicate their purification in a native conformation. Due to this reason, membrane-attached proteins have been highly underrepresented in such assays. Ex vivo detection of protein complexes may additionally result in the loss of detection of transient interactions.

2.2. Genetic approaches

In contrast to biochemical methods, in genetic assays interactions among proteins can be studied in in vivo settings and in physiological environments, thus bypassing time-consuming optimisation of conditions. The classical yeast-two-hybrid system is currently the most widely used genetic system to identify protein interactions. In this system the proteins of interest are fused to either one of two separated domains of a transcription factor, a DNA binding domain and a transcription activation domain. Reconstitution of the transcriptional activator by a particular protein - protein interaction in the nucleus results in expression of selectable reporter genes. Interactions are detected by growth selection or colorimetric assays, which are
Introduction

The yeast-two-hybrid has been used in several attempts to map the protein interactome in the bacteriophage T7, the bacterium *Helicobacter pylori*, the yeast *Saccharomyces cerevisiae* and in the metazoan nemathode *Caenorhabditis elegans*. Since interactions between two proteins are detected in the nucleus, studied proteins must enter this cell compartment. A large number of proteins however cannot be transported to the nucleus due to features such as secretory signals directing them to extracellular compartments or domains, which attach them to membranes such as transmembrane domains, myristoylation- and farnesylation signals or GPI anchoring signals. Thus, similarly to *in vitro* assays, membrane-attached proteins, which constitute about 40% of all yeast gene products, have been found to be underrepresented in large-scale two-hybrid screenings performed so far to record yeast protein–protein interactions (for review see). In order to address this problem, several genetic systems to detect protein interactions in other cellular compartments than the nucleus have been developed and shall be described here.

2.2.1 β-Galactosidase complementation

Intracystronic complementation of two mutant forms of the bacterial enzyme β-Galactosidase (β-Gal) is exploited to sense protein–protein interactions in this approach. Pairs of the inactive β-Gal deletion mutant forms Δω and Δα are able to complement one another in *trans* and assemble to an active enzyme. These two mutant derivatives, bearing only weak affinity to each other, are C-terminally fused to the proteins of interest. Dimerization of interacting partners drives dimerization of the mutant β-Gal moieties and leads to complementation of the enzymatic activity. The great advantage of this system is that β-Gal activity can be detected by the use of chromogenic substrates *in situ* and in every compartment of a cell. β-Gal complementation has been used to monitor EGF receptor chain dimerization upon treatment with a cognate ligand in live mammalian cells. A considerable limitation of this system, however, is that it is not amenable to large-scale growth selection.
screenings in eukaryotic cells since β-Gal activity does not confer a significant growth advantage to such cells.

As an alternative to this technology, fluorescence energy transfer (FRET) has been used to visualize protein interactions in situ. The limitation of this technology lies in the requirement that the fluorescing groups must be sufficiently close to each other to permit efficient energy transfer.

2.2.2. Dihydrofolate reductase (DHFR) complementation

Dihydrofolate reductase (DHFR) plays a central role in the one-carbon metabolism and is required for survival of prokaryotic as well as eukaryotic cells. E.coli DHFR is selectively inhibited by the anti-folate drug trimethoprim. In contrast, murine DHFR has a 12'000-fold lower affinity to this drug and is thus not inhibited at concentrations lethal to E.coli. Murine DHFR has been described as comprising two domains both making contact to the substrate. Pelletier et al. have shown that these, catalytically inactive separated domains, when covalently linked to an interacting domain (the GCN4 leucine zipper) reassemble in a GCN4 leucine zipper-dependent manner to form an active enzyme. Reconstitution of murine DHFR activity in E.coli rescues growth in media containing trimethoprim concentrations, which completely inhibit endogenous DHFR. It was shown that the GCN4 leucine zipper can be replaced by virtually any interacting pair of proteins including membrane protein receptors and could thus, in principle, be applied for cDNA library screenings to identify protein–protein interactions. By the use of a fluorescein-conjugated substrate of DHFR protein interactions can be directly detected in situ in any cell compartment by fluorescence microscopy or subjected to further analysis by spectrometry or fluorescence activated cell sorting (FACS). However, until now, no genetic screening identifying novel protein interactions by the use of the DHFR complementation system has been published.
2.2.3. Use of G-protein fusions to detect interactions between membrane-attached proteins

This system exploits the well-described G-protein signalling pathway as a read-out. G-protein coupled receptor activation triggers a conformational change in the G-protein α-subunit inducing exchange of GDP by GTP. Consequently Gα dissociates from the G-protein βγ-subunits. Either Gα or Gβγ-subunits activate downstream effectors until GTP is hydrolysed. In yeast, pheromone stimulation leads to dissociation of GPA1 (Gα) from the STE4 (Gβ)/STE18 (Gγ)-subunits. Gβγ leads, through a kinase-signalling cascade, to cell cycle arrest, gene transcription, cell fusion and mating. In this system a protein of interest is solubly expressed in the cytosol as a fusion protein to Gγ, whereas an integral membrane protein of interest is expressed in its wild type conformation. Interaction between the Gγ-fusion with the integral membrane sequesters Gβγ and interrupts signalling to growth arrest and mating. As a consequence cells expressing interacting partners continue to grow in presence of mating pheromones.

**Figure 1.** G-protein fusions to detect protein – protein interactions in yeast. In *Sacharomyces cerevisiae* binding of pheromones to their G-protein coupled receptors cause growth arrest. Upon pheromone binding, a conformational change in the receptor cytoplasmic tail, induces exchange of GDP through GTP in the α-subunit of the trimeric G-protein. Consequently GTP-α dissociates from the βγ-subunits until GTP is hydrolysed. Through a kinase-signalling cascade, the βγ-heterodimer causes cell cycle arrest and induces gene transcription, preparing the cell for mating. Interaction of a candidate gene product (cDNA), fused to Gγ, with a membrane-attached protein of interest (X) inhibits βγ interaction with its downstream effector, thus blocking βγ-signalling to cell cycle arrest. In this system, interaction of two proteins of interest permits growth in the presence of mating pheromones.
pheromone. In contrast to two-hybrid methods, in this system only one protein is required to be expressed as a fusion. In rare cases fusions may alter functional activity of a protein, although rather conformation than function is crucial for interaction screenings. A disadvantage compared to the membrane two-hybrid systems described below is that in this system one interacting partner must be expressed as a soluble cytosolic protein. Thus, G-protein fusions cannot detect interactions between two integral membrane proteins. Dohlman and colleagues have demonstrated the interaction between syntaxin 1 and neuronal Sec1 by applying the G-protein fusion system. In a subsequent screening they identified Sec1 mutants that are no longer able to bind syntaxin 1.

2.2.4. SOS recruitment system (SRS) / Ras recruitment system (RRS)

Both systems described in this section take advantage of the finding that some mammalian components of the ras-signalling pathway can substitute for their homologues in yeast. Ras is localised to the plasma membrane by a farnesyl moiety attached to a consensus sequence in its C-terminus. Ras on one hand is activated by guanyl nucleotide exchange factors (GEFs) and on the other hand negatively regulated by GTPase activating proteins (GAPs). Yeast cdc25 is a Ras specific GEF and exchanges GDP with GTP. Ras-GTP signalling, through andenylate cyclase, culminates in cell cycle progression. Consequently, the temperatur-sensitive cdc25-2 strain is unable to grow at non-permissive temperature. If localised to the plasma membrane, hSOS the human homologue of cdc25, can complement for the temperature sensitive allele cdc25-2 to allow cell survival and proliferation. In the SOS recruitment system (SRS) hSOS localisation to the plasma membrane in a cdc25-2 strain is mediated by interaction between two hybrid proteins of which one is fused to hSOS and the other one to a myristoylation signal that attaches it to the membrane. The SRS was successfully used to screen for negative regulators of c-Jun. In this set-up of the SRS a relatively high number of false positive clones were found to be mammalian Ras. Since yeast GAPs are unable to inhibit mammalian Ras, rare events of hSOS interactions with mammalian Ras are sufficient to rescue growth of cdc25-2 at non-permissive temperature. Co-expression of mammalian GAPs however solved
this problem. An alternative approach termed Ras recruitment system (RRS) exploits the requirement of Ras localisation to the membrane for its function.

**Figure 2. SOS recruitment system (SRS) and Ras recruitment system (RRS).** Cdc25, the yeast Ras guanyl nucleotide exchange factor (GEF) activates the Ras signalling cascade by exchanging GDP through GTP. GTPase activating proteins (GAPs) negatively regulate Ras signalling. In yeast GTP-Ras activates adenylate cyclase, which signals to cell cycle progression. Mammalian Ras pathway members can substitute for their homologues in yeast if recruited to the plasma membrane. Localisation of hSOS, the human homologue of cdc25, to the membrane rescues growth of a temperature sensitive cdc25-2 strain at nonpermissive temperature (37°C). In the SOS recruitment system (SRS) localization of hSOS to the membrane is mediated via protein interaction between a protein of interest (X) and a candidate gene product (cDNA). The Ras recruitment system (RRS), in contrast exploits the fact that Ras needs to localise to the plasma membrane to exert its function. A constitutively active mutant form of mouse Ras (mRas(61)ΔF) rescues growth at nonpermissive temperature in the same cdc25-2 strain if recruited to the membrane by protein – protein interaction.

Instead of hSOS, a constitutively active mutant mammalian Ras lacking its farnesylation signal (mRas(61)ΔF) is recruited to the membrane by protein-protein interactions. In the same cdc25-2 background protein interaction mediated recruitment of mRas(61)ΔF to the membrane rescues growth at elevated temperature.
strategy readily excludes false Ras-positives (since wild-type Ras, in cdc25-2 strains can no longer be activated) however, cannot exclude reconstitution of yeast Ras-signalling by clones expressing hSOS (hSOS would complement cdc25-2 and activate the yeast endogenous Ras). Neither the SRS nor the RRS are amenable to detect interactions between two integral membrane protein since direct fusion of integral membrane proteins to hSOS in SRS or mRas(61)ΔF in RRS would constitutively activate Ras-signalling and rescue growth at elevated temperature independent of protein interaction. Recently the RRS was adapted to study dimerization-induced autophosphorylation of the kinase domain of the EGF receptor. In this set-up Ras(61)ΔF was expressed as a fusion protein to the phospho-tyrosine adaptor Grb2. Autophosphorylation of tyrosine residues in the EGFR C-terminus recruited the Grb2-Ras fusion to the phospho-tyrosine and further activated Ras-signalling \(^{26}\). It is, however, not apparent why the membrane-attached constitutively active Grb2-Ras in this approach, can be further activated by recruitment to phospho-tyrosines. Interactions between transmembrane proteins can be studied in a similar approach, where the proteins of interest are fused to the N-terminus of the mouse EGF receptor (mEGFR). Dimerisation-induced activation of the mEGFR kinase domain and subsequent cross-phosphorylation of the tyrosine residues recruits the Grb2-Ras, lacking a farnesylation or a myristoylation signal, to the membrane and rescues growth at elevated temperature (T. Gunde and A. Barberis, unpublished results). Until present such a modification of the RRS was not implicated for a genetic screening to identify integral membrane protein interactions.

2.2.5. Mammalian protein – protein interaction trap (MAPPIT)

In mammalian cells ligand-induced clustering of type I cytokine receptor subunits leads to phosphorylation of tyrosine residues in the C-terminus of the receptor chains by receptor-associated janus kinases (JAKs). Such modified phospho-tyrosines are recognised by signal transducers and activators of transcription (STATs), which upon binding to the receptor and subsequent phosphorylation by JAKs translocate to the nucleus where they bind to specific DNA motifs in the promoter region of their target genes such as rPAP1. Mutating three tyrosine residues in the C-terminus of the leptin
receptor (LR) to phenylalanine (LR-F3) uncouples downstream signalling from receptor chain dimerization \(^{27}\). By the use of gp130 C-terminus (gp130ct), containing four STAT binding sites, Eyckerman et al. \(^{27}\) showed that foreign STAT binding sites can reconstitute ligand induced STAT signalling \emph{in trans} if recruited to the mutated LR-F3 receptor chains. Two interacting proteins, each C-terminally fused to either LR-F3 or gp130ct, mediated recruitment of gp130ct to the mutated LR in this system. Upon interaction of two proteins of interest, STAT-induced expression of a puromycin resistance gene driven by the rPAP1 gene promoter allows selection of

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{mappit.png}
\caption{The mammalian protein – protein interaction trap (MAPPIT). A) Binding of leptin (L) to its receptor (LR) causes receptor clustering and subsequent phosphorylation of tyrosine residues in the cytosolic portion of the receptor chains by receptor-associated janus kinases (JAKs). Signal transducers and activators of transcription (STATs) bind to phospho-tyrosines by their SH2 domain. JAKs in turn phosphorylate STAT monomers, which upon receptor activation, form active complexes that enter the nucleus and activate transcription of target genes. B) Point mutations exchanging the tyrosine residues in the C-terminus of the receptor chains by phenylalanine (Y→F) un Couple STAT signalling from receptor dimerization and JAK activity. Recruiting gp130ct that contains four intact STAT binding sites to receptor dimers can reconstitute STAT signalling and causes induction of transcription from a STAT target gene (rPAP1) promoter. In the MAPPIT system, interaction between protein X and a candidate gene product (cDNA) cause STAT induced expression of the puromycin resistance gene under the control of the rPAP1 gene promoter. This permits growth of mammalian cells in media containing puromycin.}
\end{figure}
clones that co-express interacting proteins by growth in puromycin containing media. In a cDNA screening using a retroviral gene transfer to attain expression from single integrants Eyckerman et al. identified the proteins CIS and SOCS-2 to be interaction partners of the phosphotyrosine 402-binding motif in the erythropoietin receptor. Thus, the authors demonstrated detection of a modification dependant protein – protein interaction, which would probably not have been identified in a comparable yeast-two-hybrid screening.

In contrast to heterologous systems, mammalian protein interactions mediated by cofactors or protein-modifications, such as phosphorylation, may preferably be detected in this system. Contrary, genes expressed in mammalian cDNA libraries may more likely interfere with the endogenous STAT-signalling pathway and thus lead to false positives. Compared to the ease of handling yeast, a screening in mammalian cells remains much more laborious and time consuming.

2. 2. 6. The split-ubiquitin system

In the split-ubiquitin system, which has originally been proposed by Johnsson and Varshavsky 28, ubiquitin serves as sensor protein. Ubiquitin is a small protein, which is covalently linked to lysine residues of proteins in order to target them for degradation by the 26S proteasome. Prior to degradation of the target protein, the ubiquitin moieties are recycled upon liberation from the target by ubiquitin specific proteases (UBPs). UBPs cleave at the junction between ubiquitin and the attached target protein; right C-terminally to the last residue of ubiquitin (for review see 29). Ubiquitin consists of two globular domains joint by a short linker. Separated N-terminal and C-terminal domains, termed Nub and Cub respectively, still reassemble and fold into a functional “split-ubiquitin”, which is recognized by UBPs as a substrate 28. Introduction of a point mutation (I13G) in Nub led to NubG. NubG and Cub do no longer reassemble spontaneously but fold into a functional split-ubiquitin if brought together. In the split-ubiquitin system proteins of interest are N-terminally fused to either NubG or Cub. Dimerization of the proteins of interest brings NubG and Cub into close proximity allowing reconstitution of split-ubiquitin and leads to UBP-conducted liberation of any effector protein fused to the C-terminus of Cub.
**Figure 4. The split-ubiquitin system to detect protein interactions.** A) Ubiquitin ligation to target proteins (T) tags them for degradation. Prior to target degradation ubiquitin is recycled by ubiquitin specific proteases (UBPs), which liberate ubiquitin from the target protein. Ubiquitin consists of two globular subunits joint by a short linker. Separated N-terminal and C-terminal domains, Nub and Cub, respectively, spontaneously reassemble to split-ubiquitin that is still recognised by UBPs. B) NubG, a mutant derivative of Nub exhibits lower affinity to Cub. NubG and Cub do no longer spontaneously assemble to split-ubiquitin. Covalent linkage of interacting proteins (X and cDNA) to either NubG and Cub can drive reassembly of separated domains to split-ubiquitin. UBP cleavage results in liberation of a C-terminally fused effector protein (E) that induces a growth selectable phenotype. C) The split-ubiquitin method has been applied to detect interactions of integral membrane proteins with cytosolic components and with transmembrane proteins.

Two effector systems have been described recently. In one a transcriptional activator acts as effector, which upon UBP cleavage translocates to the nucleus where it activates transcription of selectable reporter genes. In the second system a modified version of the URA3 gene product containing a degradation signal in its N-Terminus is C-terminally fused to Cub. The orotidine 5’ decarboxylase, the URA3 gene product (Ura3p), processes in an enzymatic reaction 5-Fluoro-orothic acid (5FOA) into a toxic product thus inhibiting growth on media containing 5FOA. Interaction caused UBP cleavage instead liberates Ura3p and thereby reveals its degradation signal. Thus, interaction of two proteins in this system leads to liberation and subsequent degradation of Ura3p allowing growth on 5FOA plates. Although the URA3 gene has been successfully used for counterselection procedures, its
application in large scale screenings in inconvenient due to the general high background of unspecific growth in the presence of 5FOA \(^{32}\) and the laborious procedure of multiple replica plating that is necessary for this selection assay.

The split-ubiquitin system has also been adapted to study and screen for interactions between membrane-attached proteins \(^{33,34}\). Depending on the orientation of the proteins of interest in the membrane, NubG and Cub are fused either N-terminally (in the case of type II transmembrane proteins) or C-terminally (type I) to the proteins of interest so that the ubiquitin moieties reside in the cytoplasm.

2.3. Screening for protein – protein interactions in a topologically extracellular compartment of yeast.

The extracellular environment differs in many aspects from the intracellular milieu. \(\text{Na}^+ \) and \(\text{Ca}^{2+} \) concentrations for example are generally higher in the extracellular space and the redox-potential is rather oxidizing to cysteine residues than reducing as it is in the cytosol. Proteins expressed on the cell surface or in a topologically extracellular compartment such as the endoplasmic reticulum (ER) or the Golgi apparatus, are adapted to prevalent conditions and additionally subjected to specific post-translational modifications that influence their biophysical properties. For instance N-linked protein glycosylation in the secretory pathway can modify protein stability and binding affinities of extracellular interaction partners \(^{35,36}\). Importantly, in the oxidizing environment of extracellular compartments cysteine residues can be covalently linked to each other through so called disulfide-bonds, which are in many cases required for proper protein folding and sustained protein stability \(^{37}\).

Considering their importance in cell regulatory mechanisms identifying interactions among extracellular proteins is of great interest. Due to the following reasons genetic systems detecting protein – protein interactions in the nucleus or in the cytosol may, however, not be adequate to detect extracellular protein interactions: i) Interactions between some cell surface receptors, \(e.g.\) integrins, and their ligands depend on extracellular concentrations of cations \(^{38}\), ii) disulfide bonds cannot form in the oxidizing environment of the cytosol \(^{39}\) and cytosolic expression of extracellular and transmembrane proteins may thus lead to misfolding and aggregation, and iii)
complex conformational binding domains of multi-spanning transmembrane proteins are unlike to be reconstituted in the cytosol.

We thus developed a novel yeast cell growth selection system, which allows detection of protein – protein interactions occurring in the topologically extracellular environment of the ER. In this system we employed components of the yeast unfolded protein response (UPR) as sensory elements for protein interactions.

2.3.1. The yeast unfolded protein response (UPR)

In eukaryotic cells accumulation of unfolded proteins in the ER triggers a stress response, which culminates in expression of stress genes, such as the ER-resident chaperone Kar2p/BIP. In contrast to higher eukaryotes, where at least three pathways contribute to UPR signalling, in yeast the type I transmembrane protein Ire1p is the only described receptor to sense accumulation of unfolded proteins in the ER lumen. Transduction of the stress signal to the nucleus in yeast is carried out by the transcriptional activator Hac1p. Ire1p comprises an N-terminal luminal domain (NLD) possessing dimerization activity, a transmembrane domain (TM) and a cytosolic portion, which contains an intrinsic serine/threonine kinase as well as an endoribonuclease in its very C-terminus. At non-stress conditions the chaperone BIP associates to the NLD and hinders Ire1p homo-dimerization. In case of UP-stress BIP preferably binds to unfolded proteins to assist their refolding and by this is titrated away from the Ire1p-NLD. The now liberated NLD homo-dimerises and brings the cytosolic portions of Ire1p monomers into close proximity, activating the intrinsic kinase. Cross-phosphorylation subsequently activates the endoribonuclease located in the Ire1p C-terminus, which in concert with tRNA ligase removes a 252-nucleotide intron of HAC1u mRNA (“u” for UPR uninduced) to produce the HAC1i mRNA (“i” for UPR induced). This unconventional splicing reaction results in a frame shift in the HAC1 mRNA open reading frame. Translation of the HAC1i mRNA results in the functional transcription factor Hac1p, which, upon activation of the UPR signalling cascade, binds to unfolded protein response element (UPREs) in the promoter region of stress genes and thereby activates their transcription. Unfolded or aggregated proteins that accumulate in the ER are
exported to the cytosol and degraded by components of the ER-associated degradation pathway (ERAD). UPR signalling activates transcription of ERAD genes such as DER1, HRD1, HRD3 or PER100 \cite{52,53}.

UPR mutant cells (\(\Delta\)ire1 or \(\Delta\)hac1) are inositol auxotroph and exhibit an increased sensitivity to UP stress inducing agents (such as tunicamycin, DTT) and elevated temperature \cite{45,46}. Deletion of ERAD genes causes constitutive activation of UPR signalling, which indicates cooperation between UPR and ERAD to overcome unfolded protein stress. Combined mutations in both, the ERAD and the UPR, cause synthetic lethality at nonpermissive temperature \cite{52}.

### 2. 3. 2. SCINEX-P (Screening for interactions between extracellular proteins)

Two mutually complementing mutant derivatives of Ire1p act a sensor for protein interaction in this system. Ire1K702R contains a point mutation in the kinase domain, which reduces its signalling potential upon dimerisation to about 40\% \cite{46}. Ire1\(\Delta\)tail bears a C-terminal deletion for its last 133 amino acids, which completely abolishes its signalling activity \cite{50}. Upon hetero-dimer formation, these mutant forms of Ire1p complement each other to attain nearly 100\% UPR signalling activity \cite{50}. The test proteins in this system are fused to either one of the Ire1p mutants such as to substitute for their NLD. Interaction between the proteins of interest brings the mutant Ire1p moieties into close proximity, which allows mutual complementation and reconstitution of the UPR signalling cascade. In order to generate an UPR reporter strain, we knocked out DER1 and/or IRE1, which are components of the yeast ERAD and UPR, respectively. In addition we stably integrated a reporter gene construct consisting of the two divergently oriented \(HIS3\) and \(lacZ\) reporter genes under the control of a synthetic bi-directional promoter that contains one, two or three UPREs. In these knockout strains, dimerisation causes reconstitution of Ire1p activity and subsequent Hac1p production. As a consequence, three independently selectable read-out systems are activated allowing growth under conditions that are selective for i) inositol synthesis, ii) resistance to higher temperature and iii) Hac1p dependent reporter gene expression \cite{54}. By applying conditions selective for different combinations of these three read-outs, the stringency of growth selection can be modulated. The feasibility of this system to monitor extracellular protein - protein
Figure 5. Screening for interactions between extracellular proteins: SCINEX-P and its potential applications. In this system interaction of two proteins of interest (X and cDNA) is detected via induced complementation of two mutant derivatives of Ire1p (Ire1K702R and Ire1Δtail). Upon dimerization of the mutant Ire1p moieties Ire1Δtail phosphorylates the kinase mutant Ire1K702R and by this activates its intrinsic RNase activity, which in concert with tRNA ligase removes an intron in the Hac1u mRNA. Translation of the processed Hac1i mRNA produces the transcriptional activator Hac1p. Hac1p in turn binds to unfolded protein response elements (UPREs) in the promoter region of endogenous stress genes and integrated selectable reporter genes (lacZ/HIS3), which promote (or inhibit) growth under selective conditions. Induced expression of endogenous genes by Hac1p suppresses the temperature sensitive phenotype of Δire1 cells. Besides this Hac1p production is important for inositol synthesis. A) Detection of interaction between two extracellular proteins in the topologically extracellular lumen of the ER. The proteins of interest are fused to Ire1p N-terminally of its transmembrane domain. B) Integral membrane proteins can be expressed as full-length proteins fused to the cytoplasmic portion of Ire1p. C) Expression of a soluble ligand into the ER causes receptor chain dimerisation and complementation of C-terminally fused mutant Ire1p derivatives.

interactions and to select for specific interactions by growth was demonstrated with specific antigen – antibody pairings in the lumen of the yeast endoplasmic reticulum. 54. SCINEX-P was then used, in two consecutive screenings rounds, to select scFv
antibody fragments that specifically bind to human Interleukin-13 (hIL-13). In the first screening scFv fragments specifically binding to hIL-13 were selected from a scFv-library. In the second round, binders obtained in the first screening were further randomised by homologous recombination in yeast cells, which were then subjected to screening conditions of higher stringency, allowing selection of binders with improved binding activity (chapter 3.2. of this thesis).
Table 1. Summary of genetic systems to detect protein - protein interactions outside the nucleus

<table>
<thead>
<tr>
<th>Detection of cytosolic protein interactions</th>
<th>Detection of integral membrane protein interactions</th>
<th>Detection of extracellular protein interactions</th>
<th>Amenable for growth selection</th>
<th>Screenings published</th>
<th>Advantages</th>
<th>Limitations</th>
<th>false positives</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Gal complementation</td>
<td></td>
<td></td>
<td>y/y</td>
<td>y/y</td>
<td>Sensor protein activity (β-Gal) is not restricted to a particular cellular compartment. In vivo visualisation of β-Gal activity.</td>
<td>Not amenable for growth selection.</td>
<td></td>
<td>11,12</td>
</tr>
<tr>
<td>DHFR complementation</td>
<td></td>
<td></td>
<td>y/y</td>
<td>y/y</td>
<td>Sensor protein activity (DHFR) is not restricted to a particular cellular compartment. In vivo visualisation of DHFR activity.</td>
<td>Growth selection only if DHFR is localized to the cytoplasm.</td>
<td></td>
<td>14-19</td>
</tr>
<tr>
<td>SRS</td>
<td></td>
<td></td>
<td>y/y</td>
<td>y/n</td>
<td>Detection of protein interactions outside the nucleus</td>
<td>Not suitable to screen for interaction partners of membrane attached protein. No reporter gene activity for signal quantitation.</td>
<td>Mammalian Ras and SOS in cDNA libraries.</td>
<td>22-24</td>
</tr>
<tr>
<td>RRS</td>
<td></td>
<td></td>
<td>y/y</td>
<td>y/n</td>
<td>Detection of protein interactions outside the nucleus</td>
<td>Not suitable to screen for interaction partners of membrane attached proteins. No reporter gene activity for signal quantitation.</td>
<td>Mammalian SOS in cDNA libraries.</td>
<td>25-26</td>
</tr>
<tr>
<td>G-protein fusions</td>
<td></td>
<td></td>
<td>y/y</td>
<td>y/n</td>
<td>Only one of the interacting proteins must be expressed as a fusion protein.</td>
<td>Prey must be solubly expressed in the cytoplasm.</td>
<td>not reported.</td>
<td>20,21</td>
</tr>
<tr>
<td>MAPPIT</td>
<td></td>
<td></td>
<td>y/y</td>
<td>y/n</td>
<td>Mammalian proteins can be expressed in their cognate environment. Higher probability to detect modification dependent protein interactions.</td>
<td>Prey must be solubly expressed in the cytoplasm.</td>
<td>not reported.</td>
<td>27</td>
</tr>
<tr>
<td>Split ubiquitin Transcriptional read-out</td>
<td></td>
<td></td>
<td>y/y</td>
<td>y/(n)</td>
<td>Allows detection of interaction between two integral membrane proteins.</td>
<td>One interacting protein must be membrane attached.</td>
<td>Ubiquitin expressing clones.</td>
<td>28-30,33,34</td>
</tr>
<tr>
<td>Split ubiquitin Ura3p enzymatic read-out</td>
<td></td>
<td></td>
<td>y/y</td>
<td>y/(n)</td>
<td>Allows detection of interactions between cytosolic, integral membrane and/or extracellular proteins.</td>
<td>No reporter gene activity for signal quantitation. Laborious and expensive growth selection on 5FOA.</td>
<td>Ubiquitin expressing clones and URA3 mutants.</td>
<td>28,29,31</td>
</tr>
<tr>
<td>SCINEX-P</td>
<td></td>
<td></td>
<td>y/y</td>
<td>y/y</td>
<td>Allows detection of interactions between cytosolic, integral membrane and/or extracellular proteins. Stringency of growth selection can be modified by combinatorial use of the three selective read-out systems.</td>
<td>Proteins with homo-dimerisation activity should not be expressed as fusions to Ire1K702R, due to its residual activity.</td>
<td>Proteins with homo-dimerisation activity if cDNA library is fused to Ire1K702R.</td>
<td>54</td>
</tr>
</tbody>
</table>

**y** = yes; **(y)** = yes but only one of the proteins of interest can be expressed as membrane attached proteins; **(y)** = manuscript submitted; **n** = no; **(n)** = need further adaptation; β-Gal = β-Galactosidase; DHFR = dihydrofolate reductase; FACS = fluorescence activated cell sorter; 5FOA = 5-fluoro-orothic acid.
2.4. References


3. RESULTS

3. 1. Part I: Cell growth selection system to detect extracellular and transmembrane protein interactions
Cell growth selection system to detect extracellular and transmembrane protein interactions

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Received 27 February 2003; received in revised form 2 June 2003; accepted 18 June 2003

Abstract

The interplay among extracellular and cell surface proteins, such as the interactions between ligands and receptors or between antigens and antibodies, is involved in a multitude of physiological and pathological phenomena. In the oxidizing milieu of the secretory pathway in eukaryotic cells, many extracellular proteins build disulfide bonds that significantly contribute to their correct folding and structural stability. Thus, conventional yeast two-hybrid interaction assays, which occur in the reducing intracellular environment, might not be adequate to detect extracellular protein–protein interactions. We have exploited the properties of yeast Ire1p, a type I endoplasmic reticulum (ER) membrane protein involved in the unfolded protein response (UPR) as a dimerization-activated receptor, to develop a novel system for the detection and study of interactions between extracellular and/or membrane proteins. In our system, named SCINEX-P (screening for interactions between extracellular proteins), proteins of interest were fused to truncated Ire1p so as to substitute its N-terminal luminal domain (NLD). Specific interaction between two partners caused dimerization of the Ire1p moiety, which, through the endogenous UPR signalling pathway, led to activation of transcription of genes that permit cell growth under selective conditions.

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Keywords: Extracellular protein; Transmembrane protein; Antibody; Ligand; Receptor; Protein–protein interaction

1. Introduction

In eukaryotic cells, proteins that are destined for the cell surface, for distal compartments or for secretion are translocated and processed in the endoplasmic reticulum (ER) and then conducted through the secretory pathway to their final location. The ER provides a unique oxidizing compartment in which a number of ER-resident chaperones facilitate the productive folding and the formation of disulfide bonds (for a review see Ref. [1]). Disulfide bonds between cysteine residues strongly contribute to structure and stability of extracellular proteins [2]. In addition, (N)-linked glycosylation of proteins in the ER is important for proper folding and can modulate the affinity of protein–protein interactions [3,4]. Considering the requirements necessary for proper folding and function within a membrane or in an extracellular environment, the conventional intracellular yeast two-hybrid assays, which occur in the reducing intracellular environment, may not be adequate to detect and study interactions between these types of proteins [5,6]. Alternative yeast genetic systems to monitor membrane protein–protein interactions have been described [7,8]. However, a broad application of these published systems to detect membrane or extracellular protein–protein interactions has not been reported yet, indicating that there might be inherent limitations of the assays. Thus, considering the importance of the role of extracellular and membrane proteins in biology and medicine, the development of additional systems with more versatile selection assays to specifically detect and study interactions between these types of proteins continues to be of interest.

Our system, called SCINEX-P (screening for interactions between extracellular proteins), is based on the properties of Ire1p, a type I transmembrane (TM) protein that resides in the yeast ER and controls the so-called unfolded protein response (UPR) [9]. This UPR is common to all eukaryotes and presumably a communication between the ER lumen and the nucleus. If proper protein maturation is impaired,
unfolded or incorrectly folded proteins accumulate in the ER. Eukaryotic cells respond to this kind of stress by stimulating transcription of genes encoding ER-resident chaperones and enzymes that assist protein folding in the ER lumen [10]. In *Saccharomyces cerevisiae*, transmission of the stress signal from the ER to the nucleus is carried out by the UPR receptor Ire1p through the induction of production of the transcriptional activator Hac1p [9]. The N-terminal luminal domain (NLD) of Ire1p acts as a sensor for the state of ER proteins and controls dimerization of this transmembrane (TM) protein [11,12]. The C-terminal cytosolic part of Ire1p harbours a Ser/Thr protein kinase, which is activated upon dimerization, and an RNase domain, which is regulated by phosphorylation. As depicted in Fig. 1A, following Ire1p dimerization, serial interactions of the activated endogenous endonuclease and the tRNA ligase remove a 252-nucleotide intron near the 3’ end of HAC1 mRNA (“u” for UPR uninduced) to produce the HAC1 mRNA (“i” for UPR induced) [13,14]. This splicing causes a change in the HAC1 mRNA open reading frame allowing synthesis of a functional protein, Hac1p [15]. Hac1p is a DNA-binding protein with homology to the leucine zipper family of transcription factors [16]. Upon activation of the UPR pathway, Hac1p binds to the unfolded protein response elements (UPREs) in the promoter region of genes encoding ER-resident proteins (such as, for example, *KAR2*) and thereby activates their expression [15,17] (see Fig. 1A).

Cells deleted for the *IRE1* gene or the HAC1 gene exhibit inositol auxotrophy and show an increased sensitivity to UP stress inducers, such as elevated temperature, reducing agents (e.g. DTT) and Tunicamycin, an inhibitor of glycosylation [18]. Two mutants of Ire1p have been described that can complement each other to reach nearly 100% of wild-type Ire1p activity; Ire1K702R [19], which contains a point mutation in the kinase domain, and Ire1Δtail, a truncated form lacking the C-terminal 133 amino acids of Ire1p, which includes the essential endonuclease active site. While the Ire1K702R point mutation reduces the signalling poten-

![Diagram](image)

Fig. 1. The unfolded protein response and its application for the detection of protein interactions in the topologically extracellular environment. (A) The yeast UPR pathway. The ER resident transmembrane protein Ire1p acts as a sensor for unfolded protein (UP) stress. UP stress induces Ire1p dimerization and activation by cross-phosphorylation. The intrinsic ribonuclease activity of Ire1p in concert with tRNA ligase removes the intron of the HAC1 precursor mRNA that contains a stop codon. In this unconventional splicing reaction, the so-called HAC1 mRNAs are processed into the HAC1 mRNA. Translation of this Hac1 isoform of HAC1 mRNA produces the transcriptional activator Hac1p, which binds in the nucleus to specific DNA sequences called UPREs, to activate transcription of genes encoding ER resident proteins, such as the chaperone Kar2p. In a different pathway, which is not yet understood, UP stress signalling is also required for inositol synthesis. AD: activation domain; DBD: DNA binding domain; P: phosphate group; UGA: stop codon within the Hac1 intron. (B) The components of the UPR pathway modified in our system are depicted in black, the endogenous components in grey. The single-chain Fv antibody fragments (scFv) were fused to the C-terminal moiety of Ire1p that carries a point mutation (Ire1K702R), while the epitope was fused to the tailless mutant Ire1p derivative. Specific interaction between the epitope and the scFv causes dimerization of the complementing Ire1p moieties and, through UPR signalling, activates transcription of the reporter genes *lacZ* and *HIS3* from a synthetic bidirectional promoter containing one UPRE.
tial of this protein to about 40% of wild-type Irelp. IrelpΔtail
shows no signalling activity on its own [20].

Genetically linked to the UPR is the ER-associated
degradation (ERAD) pathway [21,22]. Induction of UPR
signalling activates transcription of so-called ERAD genes,
whose products control the catabolism of unfolded and/or
aggregated proteins from the ER. Mutations in the ERAD
genes DER1, HRD1, HRD3 or PER100 cause constitutive
activation of UPR signalling, indicating that UPs accumu-
late in the absence of these ERAD components and that
UPR and ERAD cooperate to eliminate misfolded proteins
from the ER. Combined mutations in UPR and ERAD
pathway genes are synthetically lethal at nonpermissive
temperature [22].

The SCINEX-P system to detect interactions between
extracellular and/or membrane proteins is based on the
mechanism of Irelp activation triggered by its dimeriza-
tion, and exploits the properties of the complementing
Irelp mutants as well as the growth defects caused by
mutations in the IRE1 and DER1 genes. In our system,
proteins of interest were fused to the luminal N terminus
of truncated derivatives of the Irelp mutants bearing either
a point mutation in the kinase domain or a C-terminal
deletion. Specific interaction between two protein partners
causg dimerization of the complementing Irelp moieties,
thus activating UPR signalling through induced expression
of the transcriptional activator Haclp. We show that
interaction between two extracellular proteins is detectable
by measuring activity of reporter genes bearing upstream
Haclp-binding UPR sequences. In addition, we have
established a strong growth selection system by exploiting
the conditional synthetic lethality caused by defective UPR
and ERAD pathways. Dimerization of the Irelp chimeras
reconstituted UPR signalling and rescued growth at non-
permissive temperature. Thus, our system provides a
means for the screening of extracellular proteins such as
antibodies or orphan receptor ligands binding to their
extracellular partners.

2. Materials and methods

2.1. Yeast strains

The S. cerevisiae strains IKU1-3 and DKU1-5 were
generated starting from the strain W303a (leu2-3, -112;
his3-11, -15; trpl-1; ura3-1; ade2-1; can1-100; MATa).
The UPR reporter plasmids pDU141, pDU142 and
pDU143 carry the divergently oriented lacZ and the HIS3
reporter genes under the control of a bidirectional promoter
containing one, two, three and four tandem repeats of the
KAR2 UPR, respectively. The plasmids were linearized at the
XhoI site in the HIS3 3′-untranslated region, and stably
integrated into the his3-11 locus by homologous recombi-
nation. Correct integration was confirmed by genomic PCR
and functional assays. In these four UPR reporter strains
the IRE1 gene was deleted by replacement of the entire ORF
by the selectable marker kanMX4, generating the irelΔ
strains IKU1-3, IKU2-3 and IKU3-3. Deletion of the
DER1 gene in IKU1-3 by replacement of the ORF by the
URA3 auxotrophic marker resulted in the irelΔ; der1Δ
strain DKU1-5. The strain YPL-2 was made in a similar
way as IKU1-3 but starting from the strain JPY9 (MATa;
ura-3-52; his3A1; trplΔ63; lys2Δ385; gal4Δ11) also result-
ing in an irelΔ strain containing a bidirectional 1×UPRE-
acZ-HIS3 reporter gene stably integrated in the genome.
All gene substitutions by site-specific integration of the
selection cassette were verified by PCR analysis and func-
tional assays.

2.2. Recombinant plasmids

The integration vectors pDU141, pDU142 and pDU143
were generated by substitution of the 6xLexA binding sites
in pDE200 by one to four copies of the UPRE from the
KAR2 promoter [17]. Pairing of the two complementary
oligonucleotides with the respective sequence 5′-ctaggg-
gagctagctgattcggagcgtaca-3′ and 5′-ctgagccatcgcaga-
gagctagcgcggtcagtcg-3′ resulted in a double-strand oligonucleotide that contained 1xUPRE, an internal Spel site and was flanked by a XhoI and a SalI site. This
double-stranded oligonucleotide was inserted into
pDE200 cleaved by the endonucleases XhoI and SalI,
resulting in pDU141. In a second round, pDU141 was
opened with SpeI and SalI and the same oligonucleotide
was integrated resulting in pDU142, containing two tandem
copies of the UPRE with a 16-nucleotide spacer in between.
Repetition of this step leads to three tandem copies of the
UPRE in the promoter region of pDU143. The Irelp fusion
constructs were expressed from yeast expression vectors of
the origin YCPlac22, YCPlac33 and YCPlac111. In these
EcoRI- and SphI-treated vectors, the EcoRI/SphI fragment
from pDE Lex-VP80 was ligated so as to insert the actin
gene (ACT1), promoter (PACT1) and the GAL11 terminator.
In order to change the multiple cloning site, these inter-
mediate vectors were opened with Ncol, blunt-ended with
mungbean nuclease and subsequently cleaved by the SalI
restriction enzyme. The two hybridised oligonucleotides
harbouring the MCS (containing the unique sites XbaI,
XhoI, Stul, NotI and SalI) of the sequence 5′-ctaggg-
gagctagctgattcggagcgtaca-3′ and 5′-ctgagccatcgcaga-
gagctagcgcggtcagtcg-3′ were then integrated into the
opened vectors generating pDU10 (PACT1, ARS/CEN;
LEU2), pDU12 (PACT1, ARS/CEN; TRP1) and pDU13
(PACT1, ARS/CEN; URA3). To exchange the strong ACT1
promoter by the weaker truncated ADH and the very weak
IRE1 promoter in pDU10 and pDU12, the upstream region of
IRE1 (−1352 to −1) and the upstream region of ADH
(−414 to −1) were amplified by PCR. The PCR primer
binding to the 5′ part of the upstream regions contained a
flanking EcoRI site, whereas the downstream primers were
flanked by an XhoI site. The ACT1 promoter was then
substituted by ligation of the amplified promoter regions into the EcoRI and the XbaI site of the previously opened pDU10 and pDU12 resulting in pMH4 (PADH, ARS/CEN, LEU2), pMH12 (PADH, ARS/CEN, TRPI), pMH7 (PIRE1; ARS/CEN, LEU2) and pMH30 (PIRE1, ARS/CEN, TRPI). IRE1 DNA sequences were amplified from yeast genomic DNA by PCR with proof-start polymerase (QIAVEN) using primers that contained restriction sites at their 5’ ends. To generate the Ire1K702R point mutation, two additional primers harbouring the respective base mutations were used to amplify a 5’ fragment and a 3’ fragment of the Ire1p C-terminus. The two fragments were ligated by assembled PCR resulting in the complete Ire1 C-terminus containing the K702R point mutation. Different regions of IRE1 were amplified to generate the following Ire1 fragments: Ire1<sup>995–1115</sup>, extending from amino acid residues 496–1115; Ire1K702R<sup>995–1115</sup>, the same fragment as Ire1<sup>995–1115</sup> but harbouring a point mutation in the kinase domain; Ire1Δtail<sup>982–1115</sup>, Ire1 C-terminus extending from amino acids 495–982, lacking its very C-terminal 133 amino acids and Ire1ΔTM<sup>995–1115</sup>, lacking the NLD and the TM domain, containing the amino acids 555–1115. All the primers binding to the 5’ part of the Ire1 C-terminus contained a flanking Not1 site, the ones binding to the 3’ end contained a SalI site. The DNA sequences encoding the scFvs “AL-5”, “anti-GCN4wt”, “anti-GCN4(SS-), “X-Graft” as well as the epitope “GCN4LZ” were amplified by PCR from plasmids kindly provided by Auf der Maur [23,24]. The PCR primers binding to the 5’ of the respective sequence contained an XbaI site followed by the SUC2 signal sequence flanking the (his) tag. The sequence expressing the luminal part of Ost1<sup>1–448</sup> was amplified from yeast genomic DNA. All the primers assembling at the 3’ end of the coding sequence of the genes of interest (GOI) fused to IRE1 fragments, containing a Not1 restriction site. This allowed in-frame fusion to the Ire1 C-termini leading to the following junction: GOI-ggc ggc ggc IRE1 (Not1 site bold). The constructs containing JunLZ (amino acid residues 249–330), FosLZ (amino acid residues 133–239) or Ost1 (amino acid residues 1–448) fused to the Ire1K702R<sup>995–1115</sup> moiety were ligated into XbaI/SalI opened pDU10, the ones fused to the Ire1Δtail<sup>982–1115</sup> moiety were ligated the same way into pDU12. Constructs expressing GCN4LZ fused to Ire1Δtail<sup>982–1115</sup> were inserted into the XbaI/SalI sites of pDU12, pH28 and pMH30, whereas the scFv–Ire1K702R<sup>995–1115</sup> fusions were inserted into pH7. Integration of JunLZ fused to the wild-type C-terminal sequence of Ire1 (basepad 495–1115) into pDU10 and pDU12 resulted in pDU46 and pDU44, respectively.

2.3. Liquid β-galactosidase assay

The β-galactosidase assay in solution was performed using permeabilized cells as described [25]. Activity was normalized to the number of cells assayed. All measurements were performed in triplicate, and average values are given.

2.4. Serial dilution and spotting of yeast cells

Yeast transformations were performed according to the standard lithium acetate protocol [25]. Transformants were selected by using auxotrophic markers for the corresponding plasmids. Transformed yeast cell were grown overnight at 30 °C in drop-out medium (–Trp/-Leu). The saturated cultures were serially diluted in water (dilution factor 5) starting with a concentration of 4 × 10<sup>8</sup> cells/ml. Five microliters of each dilution was spotted on selective and nonselective plates. Nonselective plates were drop-out plates (–Trp/-Leu). The selective plates were drop-out plates lacking His, Trp, Leu with or without inositol. The selective plates contained 0, 10, 30, 60 and 90 mM 3-amino-1,2,4-triazole (3-AT). Six different dilutions of each transformant were spotted, which theoretically correspond to 20,000, 4000, 800, 160, 32 and 6 cells per spot, respectively. The plates were incubated at 25, 30 or 37 °C and scanned after 4 days.

3. Results

3.1. The principle of the SCINEX-P system

We exploited elements of the natural yeast response to unfolded protein stress (UPR) (Fig. 1A) to engineer a read-out system for the detection of protein interactions that occur in an extracellular environment such as the secretory pathway. In our system, the proteins of interest are fused to the reciprocally complementing C-terminal mutant Ire1p derivatives Ire1K702R<sup>995–1115</sup> and Ire1Δtm<sup>995–982</sup> [20] so as to substitute their extracellular N-terminal domain (see Introduction). Dimerization of the interacting partners brings the Ire1p moieties in close proximity, thus allowing complementation and UPR signalling to the cell nucleus through induced expression of the transcriptional activator Hac1p. We stably integrated a synthetic bidirectional promoter containing the Hac1p-binding site, a UPRE from the KAR2 promoter [26], controlling expression of the divergently oriented lacZ and HIS3 reporter genes (Fig. 1B). In this yeast strain, a specific interaction between two proteins fused to the described Ire1p fragments results in transcriptional activation of the HIS3 and lacZ reporter genes by Hac1p, whose expression can be detected either by measuring the enzymatic activity of the lacZ gene product or by growth selection on plates lacking histidine. A growth selection condition to detect interactions between Ire1p hybrid proteins is also achieved by exploiting the fact that mutation in the UPR, for example by deletion of IRE1 or HAC1, causes inositol auxotrophy. Moreover, as mentioned in the Introduction, the combination of mutations in the UPR and in the ERAD pathway genes is synthetically lethal.
at the nonpermissive temperature of 37 °C. For example, a double knockout of the UPR gene *IRE1* and the ERAD gene *DER1* allows cell growth at 25 °C but not at 37 °C. We reasoned that co-expression of two interacting proteins fused to the C-terminal moieties of the complementing *Ire1p* mutants in *Aire1/der1* cells would reconstitute UPR signalling and thus rescue growth at 37 °C and restore inositol synthesis. In the system presented here, dimerization of the *Ire1p* moieties, through a specific interaction between the proteins of interest, can be detected by growth selection upon incubation at 37 °C on plates lacking inositol and histidine.

### 3.2. Mutual complementation of *Ire1Δtail* and *Ire1K702R* for UPR signalling is dependent on heterodimerization

It has been shown that substitution of the *Ire1p* NLD with a functional leucine-zipper dimerization motif results in a constitutively active protein, thus indicating the necessity of *Ire1p* dimerization for UPR signalling [11]. One additional indication for activation of *Ire1p* upon dimerization comes from the observation that the two *Ire1p* mutant forms *Ire1K702R* and *Ire1Δtail* can functionally complement each other [20]. In order to test whether such genetic complementation is due to dimerization of the two mutants, we inserted the leucine-zipper of c-Jun (JunLZ) and the leucine-zipper of c-Fos (FosLZ) between a Suc2p signal sequence (S2ss) and the *Ire1p* C-terminal fragments *Ire1K702R*^495–1115^ and *Ire1Δtail*^495–982^, which still carry their TM domain (see Fig. 2, constructs a and b). Jun and Fos leucine zippers are known to form hetero- as well as homodimers. These fusion proteins were expressed from an *ARS/CEN* plasmid under the control of the constitutive *ACT1* gene promoter in the *Aire1* strain YPL-2. Induction of UPR signalling activity by the hybrid *Ire1p* derivatives was quantified by measuring expression of UPRE-lacZ reporter gene expression. As expected, the constructs containing an *Ire1Δtail* variant did not activate transcription above background (Fig. 3, lines 2–4), whereas those containing an *Ire1K702R* activated transcription to about 30% of the level obtained with a wild-type sequence of the respective *Ire1p* cytoplasmic region fused to the Jun and Fos leucine zippers (Fig. 3, lines 5–7). In contrast, the same *Ire1* mutants lacking a dimerization motif, termed ΔNLD, were unable to activate transcription of the *lacZ* gene (Fig. 3, lines 2 and 5). Co-expression of the complementing mutants containing the dimerization motifs activated reporter gene expression two- to threefold the level reached by the expression of the *K702R* point mutation alone, and almost completely restored the activity of the cytoplasmic wild-type sequence fused to JunLZ (Fig. 3, lines 9, 10 and 14), which showed similar activity as wild-type *Ire1p* induced by Tunicamycin (data not shown). Co-expression of the complementing mutants lacking the leucine zipper sequences induced reporter gene expression only very weakly (Fig. 3, line 8). In an additional control experiment, we expressed the luminal part of Ost1 (Ost1^11–448^), an ER resident type I TM protein, fused to either *Ire1K702R* or *Ire1Δtail* together with the *Ire1p* constructs mentioned above. For unknown reason, expression of Ost1 fused to *Ire1Δtail* per se generally caused slight reduction of β-galactoside activity. Nevertheless, our results clearly show that expression of

![Fig. 2. Schematic representation of the *Ire1p* derivatives used in this study. The Suc2p signal sequence (SS) followed by the protein of interest (X), which substitutes the *Ire1p* signal sequence (SI) and the N-terminal luminal domain (NLD) of *Ire1p* (amino acid residues 1 to 495), was fused to either *Ire1K702R*, extending from amino acid residues 495 to 1115 (a), or to the C-terminal-truncated *Ire1Δtail* (amino acid residues 495 to 982) (b). In order to localize *Ire1p* derivatives in the cytoplasm of transformed yeast cells, the N-terminal part (including the signal sequence, NLD and TM domain) was substituted by the protein of interest (X) lacking a signal sequence (c). To recruit this construct to membranes, a myristoylation signal (MS) was added at the N-terminus (d). In this study, the sequences of JunLZ, FosLZ, Ost1, anti-GNC4w, anti-GNC4(S53), A1-5, λ-GalZ and GNC4LZ were cloned in frame at the position indicated by "X". No insertion at this position resulted in *Ire1p* derivatives that lacked either the NLD only (ΔNLD in Figs. 3 and 4) or, in addition to the NLD, the signal sequence as well as the TM domain (see Fig. 6).](image)
Fig. 3. Reciprocal complementation of the two mutant Ire1p derivatives depends on their ability to form heterodimers. To the N terminus of both, the Ire1K702R<sup>995–1115</sup> and Ire1Δtail<sup>995–982</sup>, the following test proteins were fused: JunLZ, FosLZ, Ost1 and ΔNLD, which corresponds to the Suc2p signal peptide. The yeast strain YPL-2 was transformed with ARS/CEN plasmids expressing the respective constructs from an ACT1 promoter. The potential of these constructs to activate UPR signalling was determined by measuring the enzymatic activity of the lacZ reporter gene product. The control plasmids pDU44 and pDU46 express JunLZ fused to the wild-type sequence of Ire1<sup>995–1115</sup>.

Ost1<sup>1111–1448</sup> fused to one of the Ire1 mutants together with JunLZ fused to the complementing mutant did not result in an increased lacZ expression level (Fig. 3, lines 3, lines 11, 12, 13), thus indicating that specific dimerization, and not just overexpression of the Ire1p derivatives, leads to the synergistic effect of the mutual complementation of the Ire1Δtail and Ire1K702R mutants.

3.3. Detection of interaction between single-chain antibodies and their antigen by the SCINEX-P system

To further evaluate the system we took advantage of the well-characterized interactions between three different single-chain antibodies fragments (scFv) (“anti-GCN4wt”; “λ-Graft”; and “anti-GCN4(ΔS-)”); a cysteine-free variant of anti-GCN4wt [23]) and their epitope, the leucine-zipper of the yeast transcription factor Gcn4p (GCN4LZ). As described by Wörn et al. [23], the “anti-GCN4wt” has the highest affinity for the GCN4LZ epitope, with a \( K_d \) value of \((4.4 \pm 0.1) \times 10^{-11}\) M. This is followed by the so-called λ-Graft, with a \( K_d \) of \((3.8 \pm 0.8) \times 10^{-10}\) M. The affinity of “anti-GCN4(ΔS-)” for the GCN4LZ was not measurable in vitro because this scFv was extremely prone to aggregation after purification and subsequent refolding [23]. Such behaviour is most likely due to the inability of the cysteine-free “anti-GCN4(ΔS-)” to form disulfide bonds, since the four conserved cysteine residues have been mutated to either valine or alanine. By measuring the onset of denaturation in vitro, the “λ-Graft” has been shown to be more stable than the “anti-GCN4wt”, even though both antibodies can form disulfide bonds in oxidizing environment [23]. Consistent with these results, in conventional intracellular yeast two-hybrid assays for antigen–antibody interactions, the more stable “λ-Graft” performed best, whereas the “anti-GCN4wt” only showed very weak activity (about five times weaker reporter gene activity than λ-Graft) [24,27]. Such difference between these two scFv forms has been explained with their inability to form disulfide bonds in the reducing intracellular environment, and with the possibility of the “λ-Graft” to compensate for the lack of disulfide bonds with noncovalent intrachain interactions. The mutated “anti-GCN4(ΔS-)” was also not functional in this two-hybrid assay. To test the SCINEX-P system, these various anti-GCN4LZ single-chain antibodies were fused between the Suc2p secretion signal peptide (S2ss) and the C-terminal moiety of Ire1K702R<sup>995–1115</sup>, while the GCN4LZ epitope was fused between S2ss and Ire1Δtail<sup>995–982</sup> (Fig. 2). The choice of the Ire1Δtail<sup>995–982</sup> fragment for the GCN4LZ was made to prevent activation of the UPR signalling cascade by the strong homodimerization activity of GCN4LZ, which would do so if fused to the partially active Ire1K702R<sup>995–1115</sup> or to a wild-type Ire1p sequence.

To minimize nonspecific interactions due to overexpression of the Ire1K702R<sup>995–1115</sup> hybrid proteins, we expressed the scFvs from the very weak REI1 promoter, which is about seven times weaker than the Spkl-truncated ADH promoter and as much as 140 times weaker than the ACT1 promoter (data not shown). In contrast, the silent GCN4LZ-Ire1Δtail<sup>995–982</sup> hybrid protein could be expressed from the strong ACT1 promoter. The potential of the scFvs to bind GCN4LZ, thereby causing interaction of the complementing Ire1p C-terminal moieties and activation of the UPR signalling cascade, was first tested by measuring expression of the lacZ reporter gene under the control of 1 × UPR. None of the constructs showed any activity if expressed alone (Fig. 4, lines 2–6). The “λ-Graft” strongly activated reporter gene expression when co-expressed with its epitope (Fig. 4, line 7). In control experiments for the specificity of the antigen–antibody interactions we employed the scFv AL-5, which does not bind the GCN4LZ epitope, neither in vitro nor in vivo. This antibody fused to Ire1K702R<sup>995–1115</sup> showed only a very low level of UPR signal induction when co-
Screening for extracellular protein interactions in yeast

Results, Part I

Fig. 4. Epitope-antibody interaction-dependent activation of UPRE reporter gene. The S. cerevisiae strain DIKU1-5 was transformed with ARS/CEN plasmids expressing the Gcn4p leucine zipper epitope (GCN4LZ) fused to Ire1ΔTAIL495–982 and the different single-chain Fab antibody fragments (scFvs) "α-Graft", "anti-GCN4wt" (α-GCN4wt), "anti-GCN4SS(-)" (α-GCN4SS(-)) and "AL-5" fused to Ire1ΔTAIL495–982. The gene for the epitope-IRE1ΔTAIL495–982 fusion protein was expressed from a constitutive and strong ACT1 promoter, while the genes encoding the -IRE1ΔTAIL495–982 fusions were under the control of the weak IRE1 promoter. Binding of the various scFvs to the epitope was indirectly detected by measuring their ability to induce UPR signaling, and thus activate lacZ reporter gene transcription, which was under the control of one UPRE (unfolded protein responsive element). Reporter gene expression was quantified by measuring the enzymatic activity of β-Galactosidase. Expression of either the epitope or the scFvs alone did not result in significant reporter gene induction. Co-expression of the epitope with the specific GCN4LZ binders "α-Graft" or "anti-GCN4wt" strongly induced reporter gene expression. The nonspecific "AL-5" and the mutated "anti-GCN4SS(-)" only slightly activated the system when co-expressed with the GCN4LZ hybrid protein.

expressed with the GCN4LZ-Ire1ΔTAIL495–982 hybrid protein (Fig. 4, line 8). The hybrid bearing the “anti-GCN4wt” scFv, which is known to have a high binding affinity for GCN4LZ, strongly induced reporter gene expression, while the performance of the cysteine-free “anti-GCN4SS(-)" in the system was similar to that of the unspecific AL-5 (Fig. 4, line 10). Since the formation of disulfide bonds in the oxidizing environment is a prerequisite for proper folding of the characteristic immunoglobulin domains, the mutations of “anti-GCN4SS(-)" may impair the conformational stability of this scFv and thus abolish binding to the epitope. In contrast, the wild-type “anti-GCN4" performs in our extracellular system as well as the more stable “α-Graft". Although the level of protein expression from the weak IRE1 promoter was too low to allow detection by Western blot analysis of yeast extracts, the level of the various scFv hybrid proteins expressed from the stronger ACT1 promot-
er carried by the same type of vector was very similar (data not shown). Our results show that only a specific interaction between two proteins fused to the complementing mutants of Ire1p results in UPR signalling and reporter gene activation.

3.4. Only specific interactions allow cell growth on selective plates lacking histidine

To test growth selection of cells expressing two interacting proteins fused to the complementing mutants of Ire1p, we transformed the DIKU1 strain carrying the HIS3 and lacZ reporter genes under the control of 1 × UPR (see Fig. 1) with ARS/CEN plasmids expressing the scFvs α-Graft and AL-5 fusion proteins from the weak IRE1 promoter, and the GCN4LZ-Ire1ΔTAIL495–982 epitope hybrid protein from the relatively strong ACT1 promoter. For these growth assay experiments, GCN4LZ-Ire1ΔTAIL495–982 was also expressed from the weaker truncated ADH1 and IRE1 promoters. Exponentially growing cell cultures were spotted on selective plates lacking histidine, tryptophane and leucine and containing 0, 10, 30, 60 and 90 mM 3-amino-triazole (3-AT), a competitive inhibitor of the HIS3 gene product. Cells bearing empty vectors or plasmids expressing the nonspecific AL-5 scFv chimera stopped growing at 30 mM 3AT concentrations (Fig. 5A). In contrast, cells expressing the α-Graft hybrid protein together with the epitope fusion protein grew on plates containing 3-AT concentrations as high as 90 mM (Fig. 5A, and data not shown). The most pronounced effect was observed with cells expressing the GCN4LZ-Ire1ΔTAIL495–982 hybrid from the ACT1 promoter at 30 mM 3AT (Fig. 5A). Additional UPREs in the reporter gene promoter of the strains IKU2-3 and IKU3-3 resulted in stronger reporter gene activity and in increased growth rate. However, the ratio between cells expressing α-Graft and AL-5 hybrid proteins remained constant (data not shown). To avoid growth due to increased background activity of the HIS3 reporter gene in strains carrying multiple copies of UPREs, we proceeded with the strains IKU-1-3 and DIKU1-3, both containing one single UPRE in the promoter of the reporter genes.

3.5. Stringent growth selection of interacting partners on plates lacking inositol and incubated at elevated temperature

The efficacy and specificity of growth selection in the SCINEX-P system were further tested by combining the HIS3 transpositional read-out with the UPR-dependent inositol synthesis and the suppression of temperature sensitivity of ire1A der1A cells upon Ire1p dimerization. The ire1A der1A strain DIKU1 bearing the HIS3-lacZ reporter gene (Fig. 1) was transformed with ARS/CEN plasmids expressing the GCN4LZ-Ire1ΔTAIL495–982 epitope hybrid protein from the ACT1 promoter and the various Ire1K702R495–1115 single-chain fusion proteins from the
Fig. 5. Growth selection of epitope binders. Transformed yeast cells were spotted in 1:5 dilution series with a starting concentration of about 20,000 cells/spot on synthetic complete agar plates lacking histidine, leucine, tryptophane with or without inositol and 0, 10 or 30 mM 3-AT. These plates were incubated at 30 °C or 37 °C. As an epitope, cells co-expressed the leucine zipper of GCN4 (GCN4LZ) fused to the Ire1 C-terminal moiety Ire1Δtail195–202 and different scFvs ("λ-Graft", "anti-GCN4wt" (α-GCN4wt), "anti-GCN4(SS-) (α-GCN4(SS-)) and "AL-5") fused to Ire1K702R195–215. (A) IKU1-3 cells (ire1Δ) expressing the GCN4LZ-binding "λ-Graft" scFv grew on selective conditions, while cells expressing the nonspecific "AL-5" scFv were unable to grow on selective plates containing 30 mM 3-AT. The most pronounced effect was observed, when the epitope was expressed from the strong constitutive ACT1 promoter and the scFvs from the very weak Ire1 promoter. (B) DIKU1-5 cells (ire1Δ; der1Δ) expressing one of the specific binders "λ-Graft" or "α-GCN4wt" grew at every selective condition (a–f). In contrast, expression of the nonspecific "AL-5" did not rescue growth at stringent conditions (e, f). While omitting inositol, or incubation at 37 °C, had a significant negative effect on growth only in the absence of any Ire1p derivative (b and d), the combination of incubating at 37 °C and the lack of inositol synergistically increased selectivity of the system (e). Addition of 30 mM 3-AT further increased stringency (f). In contrast to the nonspecific "AL-5", which stopped growing on plates lacking inositol at 37 °C, the mutated "α-GCN4(SS-)" grew under these conditions (e and f) (see text for further explanations).

weak Ire1 promoter. Overnight cultures were spotted on agar plates. The control plates lacked histidine, leucine and tryptophane, whereas the selective plates additionally lacked inositol and contained either 0 or 30 mM 3-AT. All the plates were incubated either at 25 or 37 °C. As expected, on the control plates at 25 °C all the transformed cells grew (Fig. 5B, a). While single read-out conditions for growth selection (i.e. plates lacking inositol or incubation of nonselective plates at 37 °C) could only discriminate the empty vector controls showing some growth retardation (Fig. 5B, b and d), the combination of elevated temperature with inositol deprivation allowed a clear selection between the GCN4LZ binder λ-Graft and the non-binder AL-5 (Fig. 5B, c). Addition of 30 mM 3-
AT further enhanced the stringency of the growth selection under all tested conditions (Fig. 5B, c and f). In contrast, the anti-GCN4wt was distinguishable from the mutated cysteine-free anti-GCN4(SS) at 25 °C on 3-AT plates lacking inositol, but it was not so at 37 °C under any condition (Fig. 5B, d, e and f). We explain this phenomenon by the lack of disulfide bonds in cysteine-free anti-GCN4; although this mutation causes a change in the conformation of the immunoglobulin (Ig) domain, the protein is sufficiently stable at 30 °C (compare β-galactosidase values in Fig. 4). At the nonpermissive temperature of 37 °C, this protein might unfold and aggregate more readily, thus causing oligomerization of the Ire1K702R moiety, which has a residual but significant activity. Such oligomerization is expected to induce the UPR signalling, thus enabling growth at elevated temperature. In the ire1A der1A double mutant strain DIKU1, persistence of these aggregates, with their possible effect on UPR signalling, is expected to be higher than in DER cells because degradation of UPs through the ERAD pathway is impaired due to the deletion of the DERI gene. In contrast to the above observation with the DIKU1 strain, in the Ire1A but ERAD wild-type (DER) strain IKU1, the effect of the anti-GCN4wt—Ire1 fusion could indeed be distinguished from that of the cysteine-free anti-GCN4(SS) by growth selection at 37 °C on 3-AT plates lacking inositol (Fig. 5, g and h).

3.6. The TM domain is not necessary for UPR signalling by Ire1p

Ire1p is localized in the ER membrane (see Introduction) and signals to the nucleus the accumulation of UPs in the ER lumen. To test whether the association of Ire1p with the ER membrane is necessary for its function, we fused the dimerizing JunLZ with an Ire1p C-terminal fragment termed Ire1LTMP55–1115 that lacks the TM domain (Fig. 2). In addition, a derivative was constructed in which a myristolation signal (MS) was added to the N terminus of this fusion protein resulting in MS-Ire1LTMP55–1115. The MS sequence is expected to allow localization of the Ire1p derivatives at the membrane even in the absence of a signal peptide and a TM domain. Fig. 6 shows that both fusion proteins strongly activated Hac1p-dependent lacZ gene expression. In contrast, the same cytoplasmic Ire1p fragments lacking a functional dimerization motif showed no activity. Since the Ire1 C-terminus is able to activate reporter gene expression upon dimerization in the cytoplasm, the system presented here can also be applied to detect protein–protein interactions in the cytoplasm. Moreover, these results indicate that the C-terminal part of Ire1p lacking its TM domain could be functionally fused to the cytoplasmic end of full-length TM proteins for the SCINEX-P interaction assays.

4. Discussion

The aim of this study was to generate a novel genetic system allowing analysis of in vivo interactions that occur among proteins, or protein domains, naturally present in the extracellular environment of eukaryotic cells. The requirements necessary for proper folding and stability of such proteins (e.g. disulfide bond formation under oxidizing conditions, glycosylation, calcium concentration, etc.) are met in the secretory pathway of eukaryotic cells, from the ER to the cell surface. In the system presented here, called SCINEX-P, the luminal domain of the yeast ER-resident TM protein Ire1p was substituted with the protein of interest, thus allowing its localization in the proper environment of the ER. Although some properties of the secretory pathway in mammalian cells differ from that of yeast cells, e.g. different modification of primary glycosylation and somewhat lower calcium concentration in the ER of S. cerevisiae, the environment is essentially very similar and should allow expression of correctly folded mammalian extracellular proteins in the secretory pathway of yeast cells.

The SCINEX-P system was applied to detect specific antibody–antigen interactions, typically occurring in the extracellular environment. In our system, the well-characterized single-chain antibodies "X-Graft" and "anti-GCN4wt", both of which specifically bind the GCN4L epitope, could be clearly selected from nonspecific or destabilized scFvs, such as "AL-5" and "anti-GCN4(SS)". The fact that the "anti-GCN4wt" interacts with its epitope in our extracellular assay system but, in contrast to the ability of the "X-Graft", failed to bind in a conventional intracellular yeast two-hybrid assay [24] shows that the dissimilarities of protein folding and interaction between the secretory pathway and the cytoplasm or the nucleus can have dramatic effects on the applicability of a genetic system for protein interactions. Disulfide bonds are essential for proper folding and func-
tionality of extracellular proteins. This also applies to single-chain antibodies, unless their frameworks are extraordinarily stable even under reducing conditions because of peculiar amino acid composition, as it was shown to be the case for the "y-Graft" scFv [23]. Our results underline the importance of expressing extracellular proteins and protein domains in a cognate environment to identify and study their interactions. Furthermore, in a potential application of the SCINEX-P system, the compartmentalization of the ER could facilitate identification of novel soluble receptor ligands expressed in the ER by their ability to induce dimerization of two receptor chains fused to I re1 p. Indeed, the ER compartment would not allow massive diffusion of the ligand outside the cell, which would make it otherwise difficult to set efficient selective conditions for discriminating the individual clone from the background population.

By making use of the mutually complementing I re1 p mutants I re1 K702R, which contains a point mutation in the kinase domain, and I re1 Δtail, which lacks a C-terminal region containing the essential endonuclease activity, we circumvented the problem of UPR induction through potential homodimerization of an interaction partner. In our system, proteins that form homodimers are fused to the I re1 Δtail495–582 moiety; thus, only their interaction with a partner fused to I re1 K702R495–1117 leads to activation of the UPR signalling cascade, and thus to a positive selection of the interaction. For example, these mutually complementing I re1 p hybrids allowed detection of antibodies that bind homophilic proteins fused to I re1 Δtail495–582, such as the leucine-zipper of Gcn4p used in this study.

In order to establish a cell growth selection system, which would greatly facilitate the application of SCINEX-P in a screening assay, we established three different readout systems, which can either be applied individually or in combination: (i) Hac1p-dependent activation of leu2 and His3 reporter genes allowing growth selection on plates lacking histidine and supplemented with variable concentrations of 3-AAT; (ii) suppression by activated I re1 p hybrid proteins of the temperature sensitivity of I re1 A der1 A cells, which are defective in the UPR and in the ER-associated protein degradation (ERAD); (iii) suppression by activated I re1 p hybrid proteins of the inositol auxotrophy of I re1 A cells. It is possible that exposing cells to stressful conditions such as incubation at 37 °C and inositol depletion might activate signalling pathways interfering with the UPR readout. However, our results show that selection of specific interacting proteins from non-binders was successful in every combination of these three read-out options without the appearance of interaction-independent cell growth. Our data indicate that different combinations of these three read-out systems, including varying the concentration of 3AT, allow to establish discrete levels of stringency, which would be important to select for improved or high affinity partners from a pool of binders. We believe that such versatile selection system, which not only employs selectable reporter genes but also specific endogenous pathways, provides an advantageous alternative to the existing genetic methods [7,8] to detect membrane protein–protein interactions.

Acknowledgements

We thank Dr. Howard Riezman, Tea Gunde and Stefan Tanner for stimulating discussion, and Drs. Claudia and Florian Schärer for materials.

References


3. 2. Part II: *In vivo* screening of single-chain Fv antibody fragments against human Interleukin-13 in a topologically extracellular compartment of yeast
In vivo screening of single-chain Fv antibody fragments against human Interleukin-13 in a topologically extracellular compartment of yeast

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Running tile:
Antibody selection by an extracellular two-hybrid system
3. 2. 1. Summary

Single-chain antibody fragments (scFv) binding to full length mature cytokine Interleukin-13 (IL-13) were selected *in vivo* by using the yeast growth selection system SCINEX-P (*screening for interactions between extracellular proteins*) \(^1\). In this system, scFv fragments and human IL-13 are expressed in the topologically extracellular lumen of the endoplasmic reticulum (ER), where, due to oxidizing conditions, disulfide bonds can form. Interaction between antigen and antibody, both fused to either one of two complementing derivatives of the dimerization-activated receptor Ire1p, induces signalling to the nucleus and thereby expression of selectable reporter genes. In a primary screening, binders against IL-13 were selected from an scFv-library containing 7 randomised amino acid residues in the complementarity-determining region 3 (CDR3) of their heavy chain. The CDR3 of the light chain of these selected binders were subsequently randomised by homologous recombination *in vivo*. The resulting libraries were screened under increased selective pressure to identify binders with improved binding affinity. ScFv fragments obtained with this method specifically bind to human IL-13 in western blot assays and ELISA.
3. 2. 2. Introduction

The availability of entire eukaryotic genome sequences has fuelled endeavours to untangle the complex network of interactions between the gene products of a given organism. Identifying physical interactions of newly discovered gene products with known proteins facilitates their functional characterization. Traditional biochemical methods such as co-immunoprecipitation or cross-linking need laborious and time-consuming optimisation of conditions for every pair of proteins and are thus not amenable to high-throughput applications. For this reason, genetic systems such as the yeast-two-hybrid technology, which allows detection of protein–protein interactions in vivo and has been shown to be suitable for high-throughput screenings, have become very popular. However, conventional two-hybrid systems have evident limitations. For example, although roughly 40 percent of all proteins are anchored to the membrane extracellular and membrane-attached proteins are significantly under-represented in large-scale yeast two-hybrid screens performed so far. This indicates that conventional nuclear yeast-two-hybrid systems are not adequate for every class of proteins. Several genetic systems have been developed to study protein–protein interactions outside the nucleus such as the Split-ubiquitin system, a G-protein coupled system, the SOS recruitment system, the β-Gal complementation and DHFR complementation system and, in order to circumvent the poor performance of transmembrane and extracellular proteins in the cytoplasm, some have successfully been adapted to study and screen for interactions among integral membrane proteins. However, to our knowledge, none of these methods have been adapted to screen for extracellular protein interactions. Many extracellular proteins differ from intracellular proteins in that they are N-glycosylated and contain disulfide-bonds, which, for their formation, require an oxidising environment and cannot form in the reducing milieu of the cytoplasm or the nucleus. Other high-throughput methods, in which oxidising conditions can be reconstituted, such as phage-display and ribosomal-display, rely on protein purification. This requirement is a considerable disadvantage regarding proteins that contain transmembrane domains or disulfide-bonds, since these features complicate recombinant expression and purification. In general, methods that require protein purification show clear limitations regarding the analysis of transmembrane proteins. For example in a recent systematic analysis in
which yeast protein complexes have been purified and subsequently analysed by mass spectrometry, only 1% of identified complex members were membrane proteins 20.

We recently published a yeast growth selection method to identify interactions between transmembrane and extracellular proteins or protein domains in vivo 1. In the method termed SCINEX-P (screening for interactions between extracellular proteins), we employed components of the yeast endogenous unfolded protein response (UPR) to detect interactions between extracellular proteins in the topologically extracellular milieu of the ER lumen. In the wild-type situation, the type I transmembrane receptor Ire1p is localised to the ER membrane where it senses accumulation of unfolded proteins 21-23. If proper folding of proteins is impaired, dimerization of Ire1p is induced. Ire1p dimerization activates the UPR signalling cascade, which, through induced expression of the transcriptional activator Hac1p, signals to the nucleus 24-26. Expression of UP-stress genes induced by Hac1p confers tolerance to stress conditions such as elevated temperature 27. In addition, Hac1p is required for inositol synthesis 28. In our system the divergently oriented reporter genes lacZ and HIS3 that are under the control of a Hac1p inducible promoter, are integrated in a Δire1 yeast strain. The proteins of interest are fused to mutually complementing mutant derivatives of Ire1p 29,30 such as to substitute for the Ire1p ER-lumenal domain. Dimerization of two interacting partners in the ER lumen brings the mutant Ire1p moieties into close proximity, thus allowing complementation and UPR signalling. This permits growth under conditions that are selective for inositol synthesis, resistance to higher temperature and Hac1p reporter gene expression 1.

In the study presented here we demonstrate the suitability of the SCINEX-P system for screening purposes. As a model system we have chosen the interaction of an antibody fragment with an extracellular protein, the Interleukin-13. Both the scFv and IL-13 contain disulfide bridges, which are essential for their proper folding. The mature hIL-13 is a secreted 14kD four-helix bundle cytokine that contains four potential N-glycosylation sites and two intramolecular disulfide bridges. hIL-13 is a critical Th2-type cytokine and has been associated with immune defence against parasites via induction of IgE secretion and production of eosinophila. In the asthmatic lung however IL-13 seems to be the crucial cytokine to cause mucus hypersecretion and airway hyper responsiveness (AHR) (for review see 31), thus representing an interesting target for asthma therapy. We selected scFv fragments that
specifically bind to hIL-13. Two consecutive rounds of CDR randomisation directly in yeast, using the SCINEX-P screening technology, identified such scFv fragments. Thus, the SCINEX-P screening system was successfully used for identification of specific interactions between two extracellular proteins.
3. 2. 3. Experimental procedures

Yeast strains

The *S. cerevisiae* strains IKU1-3 and IKU3-3 are of the following genotype: *leu2-3, -112; his3-11, -15::lacZ-1xUPRE-HIS3*⁺; *trp1-1; ura3-1; ade2-1; can1-100; MTA1a*, and *leu2-3, -112; his3-11, -15::lacZ-3xUPRE-HIS3*⁺; *trp1-1; ura3-1; ade2-1; can1-100; MTA1a*, respectively. Generation of these strains was described earlier ¹.

Recombinant plasmids

Ire1p fusion constructs were expressed from yeast Ars/Cen expression vectors of the origin YCpLac22 and YCplac33. In the EcoRI- and SphI-treated vector YCplac33 the EcoRI/SphI fragment from pDU12 ¹ was ligated so as to bring in the actin gene promoter (PACT1) and the GAL11 terminator flanking the MCS (containing the unique sites XbaI, XhoI, StuI, NotI and SalI) resulting in pDU13. In the XbaI-treated pDU13 a double-stranded DNA oligonucleotide coding for the SUC2 signal sequence (aa1-19) (S2ss) was ligated such that the 5’ XbaI site was destroyed while the 3’ XbaI site was reconstituted. In the resulting vector the C-terminally truncated Ire1p derivative Ire1Δtail⁴⁹⁵-⁹⁸² (extending from residue 495 to 982) (described in ¹) was ligated such as to substitute for the NotI/SalI fragment resulting in the “bait vector”. Human IL-13, extending from amino acid 35 to 146, was amplified by PCR from a human mixed tissue library (Clonetech) with primers providing a 5’ XbaI site and a 3’ NotI site, allowing in frame cloning of hIL-13 into the XbaI- and NotI-treated “bait vector” resulting in a fusion of the S2ss, the adjacent hIL-13³⁵-¹⁴⁶ and the Ire1Δtail⁴⁹⁵-⁹⁸², in pDU172. The linkers containing the restriction sites were of the following sequence; S2ss-GCA TCT AGA GGC- hIL-13³⁵-¹⁴⁶ AAC AGC GGC CGC AAG-Ire1Δtail⁴⁹⁵-⁹⁸² (XbaI and NotI restriction sites underlined. The last codon of the preceding and the first codon of the adjacent gene are in bold letters). The library vector was constructed on the basis of pMH30 ¹, a vector of the YCplac22 origin, containing the IRE1 gene promoter and the same MCS as pDU12. pMH30 was treated with XbaI and SalI, and the released XbaI/SalI fragment was replaced by a fusion of the following three fragments in a four-way ligation: i) a double-stranded DNA
oligonucleotide coding for the S2ss flanked by a 5’ XbaI site and a 3’ SfiI site, ii) a scFv antibody framework of the type $\lambda 7$ $V_H$1 linked by a 20-mer linker (GGGGSGGGGSGGGSSGGGS). This framework flanked by distinct SfiI sites allowing directional cloning, was subcloned from pLib1 and iii) the Ire1p derivative Ire1K702R$^{526-1115}$. The linkers containing the restriction sites were of the following sequence: S2ss-GCA TCG GCC CAG CCG GCC ATG-scFv-TCG GCC TCG GGG GCC TCT- Ire1K702R$^{526-1115}$ (SfiI restriction sites underlined. The last codon of the preceding and the first codon of the adjacent gene are in bold letters). The Ire1K702R$^{526-1115}$ (bearing a point mutation in the kinase domain and extending from amino acid residue 526 to 1115) was amplified by PCR as described in earlier publication$^1$ with a 5’ primer containing an SfiI site matching to the 3’ SfiI site of the scFv fragment, and a 3’ primer flanked by a SalI site. The resulting library vector was termed pDU162. The sequences of all the constructs used in this study were verified by Sequencing.

Making of the libraries

In order to obtain the primary scFv library an existing library was subcloned into the SfiI-treated pDU162. The original library on the basis of a $V_j$3- $V_H$1 scFv fragment termed FW7.3$^{32}$, in which the 7 amino acid residues H97, H99, H100, H100A, H100B, H100C and H100D (according to Kabat numbering scheme$^{33}$) of the 15 amino acids comprising CDRH3, have been randomised, had an estimated complexity of $\sim 1 \times 10^7$ independent clones. Upon subcloning of this library into pDU162 and subsequent transformation into E.coli (KC8) about $7.5 \times 10^6$ colony forming units (cfus) were obtained. For the affinity maturation screening, the binders from the primary screening, S1-5 and S1-18, were prepared by introducing a second Acc65I site right behind the CDRL3 in addition to the existing Acc65I site directly upstream of the CDRL3. Cleavage with Acc65I removed the CDRL3. Treatment of the linearised vector with Klenow polymerase and subsequent religation of the vector led to the formation of a novel SnaBI site in the resulting CDRL3 deleted vectors. A donor DNA was synthesized by PCR using pDU162 as a template. The 3’ primer contained randomised codons for amino acid residues L93, L94, L95 and L95A (Kabat numbering scheme) in the CDRL3. The resulting PCR product started 42 bp
upstream and ended 42 bp downstream of the CDRL3, thus providing 5'/3'-flanking homology to the vector. The linearised vectors where co-transformed together with the double-stranded donor DNA into the reporter strain IKU3-3 where homologous recombination occurred in vivo. Homologous recombination between the linear vector and the donor DNA substituted the previously removed CDRL3 by the randomised CDRL3 from the donor DNA and re-circulated the vector. Randomised codons in the primer were of the sequence 5′-nnk nnk nnk nnk-3′, thus reducing the probability for stop codons from \( p = 0.047 \) to \( p = 0.031 \). As a consequence of this strategy the amino acid methionine was over-represented, whereas isoleucine was under-represented.

**Growth selection and media**

The competent *S. cerevisiae* strains IKU1-3 and IKU3-3 were pre-transformed with the “bait” construct prior to library transformation. In the primary screening, cells transformed with the library plasmid were directly plated on selective plates containing synthetic complete medium \(^3\) lacking uracil, histidine, leucine and inositol (SC -U/H/L/Ino) and containing 30mM 3-amino 1, 2, 4-triazol (3AT), incubated at 30°C for 2 days and then shifted to 37°C for additional 2 days. In the affinity maturation screening in contrast, transformed cells were first recovered in liquid SC-Ino at 30°C for 2 hours, washed in dH2O, re-suspended and plated on SC -U/H/L/Ino plates containing 30mM 3AT. Screening plates were immediately incubated at 37°C. In both screening upcoming colonies were re-streaked on SC -U/H/L/Ino + 30mM 3AT plates and incubated at either 30°C (primary screening) or 37°C (affinity maturation screening). Screening positives were selected for loss of bait plasmids by growth on SC plates containing 50μg/ml uracil and 1mg/ml 5-Fluoro-orothic acid (5FOA) at 30°C. Subsequent replica plating or filter lifting on X-Gal plates (0.2mg/ml) revealed bait-dependency. SC medium lacking inositol was prepared with inositol free yeast nitrogen base (Q-BIOgene).

**Assay of cellular \(\beta\)-Galactosidase activity**

\(\beta\)-Galactosidase activity of cells was determined using the protocol described in \(^3\)4
Protein expression and purification

In vitro analysis of the different scFv fragments was performed with purified proteins, expressed in bacteria. The screening derived scFv fragments S1-5, S1-18, S2-13, S2-14, S2-22 and S2-24 were cloned into the expression vector pAK400, providing a C-terminal His$_6$-tag, and were periplasmically expressed in _E.coli_ JM83 (λ-, _ara_, Δ(lac, proAB), _rpsL_, _thi_, Φ80, _dlacZΔM15) at 25°C. Periplasmically expressed scFv fragments were purified by immobilized metal ion affinity chromatography (making use of the C-terminal His$_6$-tag). Fractions were analysed by SDS gel-electrophoresis on a 15% polyacrylamid gel. Purified proteins were concentrated on Microcon$^R$ YM-10 columns (Millipore).

In vitro analysis

ELISA: 96 well plates (Nunclone maxisorb) were coated overnight with 2.5 μg/ml recombinant human IL-13 (research diagnostics inc. RDI) in TBS and subsequently blocked with TBST containing 5% milk. Purified scFv fragments were added to the wells at 5, 10 and 25 μM concentrations. Binding of the different scFv fragments to hIL-13 was detected with a secondary mouse anti-His6 antibody (QIAGEN) (diluted 1:500) and a tertiary goat anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP) (Santa Cruz Biotechnology) (1:4000). Signal detection occurred via measuring HRP activity using the “BM blue POD Substrate” (Roche) and measuring the optical density at λ=450 nm. Immunoblotting: 0, 4.5, 45 and 450ng recombinant human IL-13 (RDI) and 450 ng purified BSA (New England Biolabs NEB) were loaded in quadruplets on a SDS-15% PAGE gel and electroblotted on a nitrocellulose membrane (Schleicher & Schuell). After blotting, the membrane was cut into stripes each containing a set of dilutions of hIL-13 and BSA. Blot stripes were blocked with TBST + 5% milk and each incubated with one of the purified scFv fragments at a concentration of 3.0 μM or with a rabbit polyclonal anti-hIL-13 (Abcam) (diluted 1:2000) as a positive control. All the following incubation and washing steps with the different membrane slices were performed with identical volumes, concentrations and time periods. Bound antibody fragments were detected by using a secondary mouse
anti-His$_6$ antibody (1:500) and a tertiary goat anti-mouse IgG antibody conjugated to HRP (Santa Cruz Biotechnology) (1:4000), the positive control antibody was detected with a secondary anti-rabbit IgG HRP conjugated antibody (SIGMA) (1:400). Visualisation occurred with SuperSignal® West Pico Chemiluminescent Substrate (PIERCE) and x-ray films (Kodak). Antibodies were diluted to appropriate concentrations in TBST containing 0.5% milk.
3. 2. 4. Results

*In vivo screening of single-chain antibody fragments binding to human IL-13*

The SCINEX-P system allows detection of interactions among extracellular proteins occurring in the topologically extracellular lumen of the endoplasmic reticulum (ER)\(^1\). This system exploits the properties of the type I ER resident protein Ire1p, which acts as a sensor of unfolded protein (UP) stress in yeast. UP-induced dimerization of Ire1p activates a signalling cascade to the nucleus via expression of the transcriptional activator Hac1p. Hac1p activates transcription of UP stress genes, thereby conferring tolerance to stress conditions such as elevated temperature. Moreover, via largely unknown pathways, Hac1p is also required for inositol synthesis. In an IRE1-deficient yeast strain we stably integrated a synthetic bi-directional promoter containing one or three tandem copies of the Hac1p binding site, the *unfolded protein response element* (UPRE) from the KAR2 promoter\(^35\), controlling expression of divergently oriented *lacZ* and *HIS3* reporter genes. Integration of these reporter genes resulted in the reporter strains IKU1-3 and IKU3-3, respectively. In this system the proteins of interest are fused to the reciprocally complementing mutant derivatives of Ire1p, Ire1\(_{\text{Δtail}495-982}\)\(^{29}\) (extending from aa residue 495 to 982) and Ire1K702R\(_{526-1115}\)\(^{30}\) (aa 526 to 1115) so as to substitute for the Ire1p luminal domain (Figure 1A). Dimerization of two interacting partners in the lumen of the ER allows interaction of the complementing Ire1p moieties and activation of UPR signalling. In IKU1-3 and IKU3-3, UPR signalling confers increased tolerance to elevated temperatures, induced inositol synthesis and activation of reporter gene expression, thus allowing growth under three independent selective conditions: *i*) histidine depletion (the stringency of this selective pressure can be modulated by the addition of the competitive inhibitor of the *HIS3* gene product, 3-amino 1, 2, 4-triazol (3AT)), *ii*) Inositol depletion and *iii*) exposure to elevated temperatures (37°C) to which Δire1 cells are sensitive. In addition UPR signalling strength can be indirectly quantified by measuring activity of the β-Galactosidase expressed from the *lacZ* reporter gene.

As a model system to establish screening procedures we have used mature hIL-13 consisting of residues 35 to 146 (hIL-13\(_{35-146}\)). hIL-13\(_{35-146}\) was fused between the SUC2 signal sequence (S2ss) and Ire1\(_{\text{Δtail}495-982}\) resulting in the antigen-construct (Figure 1B). This construct was expressed from an *ARS/CEN URA3* plasmid under the
control of the constitutive ACT1 gene promoter. The scFv library was cloned between the S2ss and the mutant Ire1p derivative Ire1K702R²⁵⁶-¹¹¹⁵ (Figure 1. B). In this primary scFv-library, 7 residues of the complementarity-determining region 3 of the heavy chain (CDRH3) in an scFv framework of the type $V_{\lambda}^3-\text{VH}1$ termed FW7.3 were randomised. This framework has been selected from a human scFv framework library to be especially stable even in a reducing environment ³². This library was expressed from an ARS/CEN LEU2 plasmid under the control of the very weak constitutive IRE1 gene promoter (PIRE1).

**Determination of screening conditions**

The aim of this primary screening was to isolate binders from an initial scFv library, while the aim of the affinity maturation was to monitor feasibility of selecting for increased binding activity by adjusting the selective conditions. (see below). In order to find the optimal conditions we transformed the reporter strains IKU1-3 and IKU3-3 with empty vector (Figure 2a), library (b), library and antigen together (c) as well as a control construct expressed from the weak PIRE1 and consisting of the GCN4 leucine zipper (GCN4LZ), a homo-dimerization domain fused to the Ire1K702R²⁵⁶-¹¹¹⁵. GCN4LZ induced homo-dimerisation of this mutant derivative of Ire1p that still bears a residual activity, and hence induces a relatively weak UPR signal (d). This construct was chosen as positive control since its moderate signalling activity might more closely represent the signalling activity of a low affinity scFv fragment expected to be selected in a primary screening. The transformed cells were then incubated at either 30°C or 37°C for four days on synthetic complete medium selective for the transformed plasmids and containing the following modifications, each challenging different combinations of the three read-out systems outlined above and indicated in Figure 2: Depletion of inositol and incubation at non-permissive 37°C, selecting for Inositol synthesis and temperature tolerance (w); depletion of Inositol and addition of 30mM 3AT, selecting for Inositol synthesis and $\text{HIS}3$ reporter gene activation (x); addition of 30mM 3AT and incubation at 37°C selecting for $\text{HIS}3$ reporter gene activity and for temperature tolerance (y); and Inositol depletion, addition of 30mM 3AT and incubation at 37°C, selecting for all three read-out systems at once (z). For transformation and growth control, unmodified plates were incubated at the
permissive temperature of 30°C. Optimal conditions were found to be SC-U/L/H lacking Inositol and containing 30mM 3AT together with incubation at permissive temperature. With this combination of selective conditions, reasonable transformation efficiencies were obtained (d/x) whereas only very few background colonies appeared after four days (c/x). In contrast, all other conditions in which either inositol depletion or addition of 30mM 3AT was combined with direct incubation at 37°C, lowered transformation efficiency about ten fold without significantly improving the positive-to-background ratio. No significant difference in terms of selectivity was observed between the two reporter strains IKU1-3 and IKU3-3. Level of expression of the lacZ reporter gene as determined by measuring β-Galactosidase activity was generally 15-20 fold higher with the strain IKU3-3 compared to IKU1-3, facilitating detection of weakly induced reporter gene expression.

**Primary screening of the scFv-H3 library**

IKU3-3 cells expressing the hIL-13 antigen construct were transformed with the primary scFv-library. We obtained an estimated number of about 1.5 x 10⁶ colony-forming units (cfu), which were screened on selective plates lacking uracil, leucine, histidine and inositol and containing 30mM 3AT incubated at 30°C. After two days incubation about 650 colonies appeared. In addition to these fast growing colonies we observed a higher number of smaller background colonies. In order to suppress such background, we shifted the plates from 30°C to a 37°C incubator. At the non-permissive temperature, the 650 fast growing colonies continued to grow, whereas background colonies stopped growing. After day four 170 colonies were randomly picked and re-streaked on selective plates. 168 out of 170 re-streaked clones grew again on selective plates at 37°C; 26 colonies (~16%) showed an antigen dependent phenotype, since they no longer expressed the lacZ reporter gene upon loss of the URA3 bait plasmid on plates containing 5-fluoro-orotic acid (5FOA).

In order to confirm antigen-dependent and antigen-specific activation of the reporter genes, library-plasmids were isolated and re-transformed into cells expressing hIL-13 or an unrelated antigen, each fused to Ire1Δtail⁴⁹⁵-⁹⁸². Among the 9 antigen-specific clones, two were identical (only one of them (S1-18) is presented in Figure 3). Cells co-expressing the scFvs S1-5, S1-18, S1-24 and S1-26 with hIL-13 showed a
strong blue staining on X-Gal plates indicating high lacZ expression. In contrast co-expression of these clones with unrelated antigen did not significantly activate lacZ reporter gene expression, thus suggesting specific binding to hIL-13. The original framework 7.3 did not induce lacZ expression neither in the presence of hIL-13 nor in the presence of the unrelated antigen (Figure 3). Sequence analysis of the CDRH3 revealed an enrichment of cysteine-containing CDRs. Out of the nine sequenced clones, six (2/3) contained a cysteine residue in the CDRH3 (Figure 3), which is significantly more than the theoretical probability of 7/32 (codon frequency). One clone was identical to the clone S1-18 (not shown in Figure 3). One possible explanation for the prevalence of cysteines might be the formation of covalent disulfide bonds between one of the five cysteines in IL-13 and a co-expressed scFv containing cysteines in its CDRH3. Covalent linkage of the antibody to its epitope would lead to maximal UPR signalling. However, none of the binders identified in this primary screening and containing cysteine residues in the CDRH3 reached the reporter gene activity of a very strong scFv – epitope interaction such as that between the well described αGCN4 scFv and its epitope, the leucine zipper of GCN4 (data not shown). This may argue against a covalent linkage, it remains however a possibility that the covalent linkage occurs between a very low number of molecules.

In vivo affinity maturation of primary binders by homologous recombination

In order to improve in vivo binding activity of the clones resulting from the primary screening, four amino acids in the complementarity-determining region 3 of the light chain (CDRL3) were randomised. Since the cysteine residues in the CDRH3 of the clones S1-24 and S1-26 are highly disadvantageous for purification and further in vitro characterization, the affinity maturation step was performed only with the clones S1-5 and S1-18, which do not contain cysteine residues in their CDRH3 (Figure 3). We therefore replaced the CDRL3 of the clones S1-5 and S1-18 with random amino acid sequences by homologous recombination in vivo (Schärer-Brodbeck C. & Barberis A. unpublished results) (Figure 1B). For this purpose we removed the original CDRL3 by restriction digestion. A donor DNA fragment bearing the randomised CDRL3 was produced by PCR using pDU162 as a template and a 3’ primer containing the randomised sequence. The resulting PCR product started 42 bp
upstream and 42 bp downstream of the CDRL3 thus providing homologies for recombination and also spanning the randomised CDRL3 that had been removed in the target vector. The appropriate yeast strain was transformed with the linearised vector together with the double-stranded donor DNA fragments. Homologous recombination of the DNA fragment with the opened vector led to an scFv fragment containing the randomised CDRL3. The resulting libraries with a theoretical complexity of 160’000 different amino acid sequences were then subjected to a secondary screening under more stringent conditions than those of the primary screening. The transformed yeast cells were plated as in the primary screening on SC-U/L/H plates lacking inositol and containing 30mM 3AT. To select for binders with a higher affinity to the target protein than the primary binders, the stringency of the selective condition was increased by incubating the plates directly at 37°C, after a short recovery phase of 2 hours at 30°C following transformation. Under these conditions, the original binders S1-5 and S1-18 did no longer form colonies even after a long incubation time (data not shown).

Transformations of the libraries based on S1-5 and S1-18 into IKU3-3 resulted in ~880’000 and ~1’040’000 transformed yeast cells screened on selective plates, respectively. Of the approximately 500 colonies of each library that appeared after four days, a total number of 256 colonies were randomly picked and re-streaked on selective plates and incubated at 37°C. Out of the 201 colonies that grew again, 19 clones (~9.5%) showed antigen dependence upon loss of plasmid on 5-FOA plate. These 19 clones were further analysed by measuring activity of the lacZ reporter gene product in liquid after plasmid isolation and retransformation (Figure 4A). Most of the selected clones showed higher or similar lacZ reporter gene activity than the initial binder S1-18 (Figure 4A). The clones S2-13, S2-14, resulting from maturation of S1-5, and clone S2-24 derived from S1-18, stimulated lacZ reporter gene activity to more than 3-fold higher levels than the level reached with the S1-18. These β-Galactosidase values aligned with the growth coefficients of the respective clones determined in liquid cultures on screening conditions (data not shown), thus confirming that growth depends on reporter gene activity. The Ire1p derivative Ire1K702R526-1115, to which the scFv-libraries were fused, bears residual signalling potential to about 40% of the wild-type Ire1p upon formation of Ire1K702R526-1115 homodimers. CDRs comprising homo-dimerization activity would thus lead to increased UPR signalling and,
consequently, stronger reporter gene activation giving rise to a growth advantage that would not necessarily depend on binding affinity to the target protein. This possibility was examined by expressing the strongest activators from the affinity maturation screening S2-13, S2-14 and S2-24 as well as an intermediate activator S2-22 together with the IL-13 antigen construct or without in the IKU3-3 reporter strain (Figure 4B). While all binders activated \textit{lacZ} reporter gene expression when co-expressed together with hIL-13 as epitope, none of them exhibited any detectable signal when co-transformed with an empty vector. We therefore conclude that no significant homodimerization of this scFv fragment occurs in the SCINEX-P system. It is worth noting that, contrary to what we observed with the selected CDRH3 sequences described above, none of the affinity-matured binders contained any cysteine residue in the CDRL3.

Selected clones specifically bind to hIL-13 in vitro

The 19 clones obtained by the screening experiments described above display a certain potential to bind hIL-13 in the context of the SCINEX-P system. Whether this \textit{in vivo} performance is in accordance with their ability to bind hIL-13 as an epitope \textit{in vitro} was investigated with purified proteins. The scFv sequences of the original framework 7.3, the binders from the primary screening S1-5 and S1-18 as well as the affinity matured S2-13, S2-14, S2-22 and S2-24 were sub-cloned into bacterial expression vectors providing a His$_6$ tag at the C-terminus of the protein. The scFv antibodies were expressed in \textit{E. coli} and purified from extracts by IMAC. Purified scFv fragments were examined for their ability to bind to recombinant human IL-13 in ELISA. scFv fragments were added in a concentration of 5, 10 and 25 μM to 96 well plates coated with hIL-13. Bound scFv fragments were detected by a secondary anti-His$_6$ antibody, which in turn was detected by a tertiary horseradish peroxidase (HRP) conjugated antibody. Amounts of scFv fragments bound to hIL-13 were quantified by measuring HRP activity. In a preliminary ELISA all the binders delivered by the SCINEX-P system gave a signal clearly above background and three out of four binders deriving from the affinity maturation step resulted in a 2 to 3 fold higher signal compared with the primary binders (data not shown). The hIL-13 – binding activities of the scFv fragments S1-18, S2-22 and S2-24 were analysed at
various scFv concentrations (Figure 5). All three scFv antibodies specifically bound to hIL-13 in a concentration dependent manner and scFv fragment S2-24 derived from scFv S1-18 showed an increased signal compared to its precursor S1-18. None of the binders tested reached clear-cut signal saturation at the concentrations tested, indicating a rather low affinity interaction with the recombinant hIL-13 in this ELISA experiment. Nevertheless the data indicate improvement in affinity of S2-24 and to a lower extend S2-22 due to their lower ratio in signal intensity between the tested scFv concentrations, compared to S1-18. These data are in agreement with the ability, of the respective clones, to induce lacZ reporter gene expression (Figure 4B).

The ability of different scFv fragments to specifically bind hIL-13 was further examined by immunoblotting (Figure 6A). 4.5, 45 and 450 ng recombinant human IL-13 as well as 450 ng BSA for control were separated on a SDS-15% poly acrylamid gel and electroblotted. The filters were incubated with purified scFvs. As expected, the framework 7.3 did not bind to hIL-13, whereas a faint signal was detectable with the primary binder S1-18 (Figure 6 A) staining the 450 ng band of hIL-13. The affinity-matured binder S2-24 showed a clearly improved sensitivity compared to the primary binder. Indeed, the matured binder S2-24 was able to detect 45 ng of recombinant hIL-13 on a western blot (Figure 6 B). None of the binders tested in this experiment bound to BSA, thus indicating specific recognition of hIL-13. These results demonstrate that in vitro binding activities of purified scFv proteins as determined in ELISA and western blotting correlate with relative lacZ expression and growth rate of cells expressing the respective scFv antibody fragments in the context of the SCINEX-P system.
3. 2. 5. Discussion

Selecting scFv fragments binding to hIL-13

In our previous work \(^1\) we have shown that interactions between extracellular proteins can be detected with the SCINEX-P system \textit{in vivo} and that cells expressing interacting proteins can be selected by growth. In the present study, an scFv antibody library of low complexity containing seven randomised amino acids in the CDR3 of the heavy chain was screened for binders against human IL-13 using the SCINEX-P growth selection system. Binders identified in the primary screening were subsequently randomised again within a four amino acid sequence of the light chain CDR3. By increasing the stringency of the selective pressure on growth, we selected from the second randomised scFv pool those clones showing improved \textit{in vivo} binding performance as well as improved \textit{in vitro} properties, when compared to their precursors. We showed that cell growth and \textit{lacZ} reporter gene activity correlate with the relative epitope binding strength of the various scFv clones \textit{in vitro}. This is of great importance for an early discrimination between clones passing the bait-dependence test, those that have predictable higher affinities for their antigen.

Advantages of the system

\textit{In vivo} detection of interactions in a topologically extracellular environment such as the ER permits expression of extracellular or transmembrane proteins that would be incorrectly folded or unstable if expressed in the reducing environment of the cytoplasm or the of nucleus. Other methods such as phage-display or ribosomal-display, in which oxidizing conditions can be mimicked, depend on purified proteins; purification of correctly folded proteins, especially in the case of multispansning transmembrane proteins, can be extremely difficult. Since only the cytoplasmic portion of Ire1p is necessary to detect dimerization \(^1\), the mutant complementing moieties of Ire1p could be fused to the C-terminus of such full-length multispansning transmembrane proteins in order to detect interactions with other extracellular or intracellular proteins. In contrast to laborious \textit{in vitro} assays the genetic system
presented here can be readily established in any molecular biology / genetic laboratory.

**Limitations of the system**

Due to the relatively low complexity of the scFv libraries screened in this study (1.5 x 10^6 in the primary screening and about 160'000 in the affinity maturation screening), we apparently selected rather low affinity binders. The lower transformation efficiency of yeast compared to bacteria limits the maximal complexity to be screened in one step. We could show however that the system allows stepwise improvement of scFv binders by adjusting the selective pressure. Additional randomisations of other CDRs in the clones obtained in our screenings, in combination with further increased selectivity of the growth conditions, thus might lead to higher affinity antibodies. It is plausible that for example lower expression of the epitope would decrease signalling strength and thus disable growth of low affinity binders under similar conditions as those used in the preceding screening without lowering transformation efficiency.

**Further applications of the SCINEX-P system**

Complexities of eukaryotic cDNA libraries are far below the complexities described above for antibody libraries. Thus, SCINEX-P could be used to identify secreted or membrane attached proteins interacting with each other. Identification of orphan ligands binding to known cell surface receptors or vice versa is an important task in finding biological therapeutics to human disease. Expression of a soluble ligand into the secretory pathway where it meets its cognate receptor chains each fused to a complementing Ire1p derivatives would lead to ligand-induced receptor dimerization and thus to UPR signalling and growth under selective conditions. In such a set up, it would not be necessary to fuse the soluble ligand to an effector protein. Since Ire1p resides in the ER membrane, thus being restricted to a closed compartment, only cells expressing the soluble ligand but not cells in their neighbourhood would profit from a productive interaction.
3.2.6. Acknowledgments

The authors thank Prof. Dr. Howard Riezman for inspiring discussions, Dr. Peter Lichtlen for critical comments on the manuscript and Dr. Adrian Auf der Maur for material.
3. 2. 7. Figures and Figure legends

Figure 1: The principle of SCINEX-P. A) The proteins of interest are expressed as fusions to either one of the two Ire1p complementing mutant derivatives, Ire1Δtail or Ire1K70R. Upon interaction of dimerization partners, in the case depicted in this figure, antigen and a single-chain antibody fragment (scFv), the mutant Ire1p moieties come into close proximity allowing complementation and UPR signalling. Subsequent Hac1p production activates the three independently selectable read-outs: i) HIS3/lacZ reporter gene expression, ii) inositol synthesis and iii) suppression of the temperature sensitive phenotype of Δire1 cells. B) In the construct expressed in this study, the Ire1p derivatives provide the transmembrane domain (TM). As antigen the mature human IL-13 extending from amino acid residue 35 to 146 was fused between the signal sequence of the SUC2 gene (Ss) and Ire1Δtail195-982. The scFv libraries were expressed as fusions of an N-terminal SUC2 signal sequence and the C-terminal Ire1K702R526-1115. In the primary scFv library 7 amino acids in the complementarity-determining region of the heavy chain (CDRH3) were randomised. In the secondary scFv library used for affinity maturation, the light chain CDR3 (CDRL3), bearing 4 randomised amino acids, was brought in by homologous recombination of a donor DNA with the vector in vivo. The donor DNA was spanning the CDRL3 and 42 bp flanking sequence containing homology to the vector. The original CDRL3 was deleted in the selected binders deriving from the primary screening by restriction digestion prior to homologous recombination.
Figure 1

**A**

Antigen  
\[ \text{Ire1}^{\Delta \text{tail}} \rightarrow \text{Ire1K702R} \rightarrow Hac1p \]

\[ \text{HIS3} / \text{lacZ} \text{ expression} \]

\[ \text{Inositol synthesis} \]

\[ \text{suppression of temperature sensitivity} \]

**B**

Antigen  
\[ \text{IL-13}^{35-146} \rightarrow \text{Ire1}^{\Delta \text{tail}^{495-982}} \]

\[ \text{scFv-Library for primary screening} \]

\[ \text{randomized CDRH3} \]

\[ \text{scFv-Library for affinity maturation} \]

\[ \text{randomized CDRL3} \]
Figure 2: Comparison of different read-out combinations. The Δire1 reporter strains IKU1-3 and IKU3-3 were transformed with a) empty vectors, b) library plus empty vector c) library plus antigen (hIL-13) and d) a positive control leading to a moderate UPR signalling and thus mimicking a low affinity interaction between antigen and antibody. Transformations were plated on synthetic complete (SC) media selective for the plasmid markers and modified as follows to challenge different combinations of the three SCINEX-P read-out systems; v) no modification as growth control, w) lacking inositol and incubation at non-permissive temperature (37°C), selecting for inositol synthesis and temperature tolerance, x) lacking inositol and containing 30 mM of 3-amino 1, 2, 4-triazole (3AT), selecting for inositol synthesis and HIS3 reporter gene activity, y) containing 30 mM 3AT and incubation at non-permissive temperature, selecting for HIS3 reporter gene activity and temperature tolerance and z) a combination of all three read-outs. On SC medium lacking inositol and containing 30 mM 3AT good transformation efficiency (x/d) and only faint background growth (x/c) was observed.
Figure 2

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IKU1-3

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IKU3-3
Figure 3: Bait-specificity and bait-dependence of primary binders. Plasmids of screening positives from the primary screening were isolated and retransformed in IKU3-3 cells co-expressing hIL-13, an unrelated bait or an empty vector. Bait dependant lacZ reporter gene expression was monitored by X-gal filter lifts. All the scFv fragments examined in this assay showed a bait-dependant and bait-specific blue staining on X-Gal plates. The original framework 7.3 did neither bind to hIL-13 nor to an unrelated epitope. Amino acid sequences in the heavy chain CDR3 of the respective binders are indicated in single letter code. Residues randomised in the primary library are highlighted in grey; cysteine residues are depicted in bold letters. Clones S1-5 and S1-18 do not contain cysteines in the CDRH3 sequence and thus were carried on to affinity maturation (asterisks).
Figure 3

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Figure 4: Increased reporter gene activation of clones deriving from secondary screening. Plasmids of positives in the secondary screening were isolated and retransformed into IKU3-3 cells. LacZ reporter gene activity was determined by measuring enzymatic activity of β-Galactosidase, the lacZ gene product. A) scFv fragments S2-3 to S2-16 derive from S1-5 whereas scFv S2-17 to S2-27 are descending from S1-18. Most of the hIL-13 binders obtained in the second growth selection upon randomisation of the CDRL3 activated lacZ reporter gene expression to an elevated extent compared to the initial binder S1-18 when co-expressed with the hIL-13 antigen. B) The scFv fragments S1-18, S2-13, S2-14, S2-22, S2-24 strongly induce lacZ reporter gene expression when co-transformed with hIL-13 but do not if co-transformed with an empty vector (vec.). Neither the antigen fused to Ire1Δtail<sup>495-982</sup>, nor any of the scFv fragments fused to Ire1K702R<sup>526-1115</sup> showed any reporter gene activation by itself. Amino acid sequences in the light chain CDR3 of the respective scFv fragments are indicated.
Figure 4

A

B

\(\beta\)-Gal. units

\(\beta\)-Gal. units

V\(_L\)-CDR3: QTAG RRRR RGQG PHYP SSSD

S2-13 S2-14 S2-22 S2-24 vector S1-18
Figure 5: Antigen recognition of purified scFv fragments in ELISA. The scFv fragments S1-18 and derivatives thereof S2-22 and S2-24 were expressed in *E.coli* and purified by IMAC. Qualitative binding properties of the purified scFv fragments were analysed in ELISA. Purified scFvs were added in different concentrations to either uncoated wells or to wells coated with 2.5 µg/ml recombinant hIL-13. S1-18, S2-22 and S2-24 bound to hIL-13 in an antigen- and scFv-concentration dependant manner. The signal of the scFv fragment S2-24 at 10 µM concentration was about 3 fold stronger than the one of its precursor S1-18.
Figure 5
Figure 6: Antigen recognition of purified scFv fragments in western blot. 4.5, 45 and 450 ng of recombinant hIL-13 and 450 ng of BSA were separated on a SDS-15% polyacrylamid gel and electroblotted on a nitrocellulose membrane. Blots were incubated with either purified scFv fragments at a concentration of 3.0 µM or with a polyclonal anti-hIL-13 antibody as a control. Bound scFv fragments were detected via their C-terminal His₆-tag. A) While the original framework 7.3 did not exhibit any binding activity to hIL-13 the primary screening positive S1-18 weakly stained the 450 ng hIL-13 band. The improved S2-24 showed a clearly enhanced signal compared to S1-18. B) With optimised washing and incubation periods S2-24 detected 45 ng of recombinant hIL-13.
## Figure 6

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### 3.2.8. References


3. 3. Part III

3. 3. 1. Counter selection in the SCINEX-P system

The identification of novel protein – protein interactions is an important step in the process of characterisation of a gene product function and its potential implications in disease. Once identified, disease-relevant protein interactions are promising targets for therapy by antibodies or small molecules that are able to break such contacts. So-called counter selection genetic systems can be used to screen gene and compound libraries for identifying molecules that specifically block a certain protein pairing. In contrast to the classical yeast two-hybrid system, in which a protein interaction causes transcriptional activation of a reporter gene that confers a growth advantage to the respective cell, in counter selection systems expression of reporter genes inhibits growth, thus allowing positive selection screening for factors or substances that disrupt a certain protein interaction and block transcriptional activation. In addition, negative selection systems can be used to identify loss of function mutations that impair binding to the target protein. Sequence analysis of such mutants can reveal limiting residues involved in a certain protein interaction and thus enable characterisation of specific protein pair domains. In contrast to genetic systems, biochemical methods such as phage-display and ribosomal-display cannot readily be used for the identification of loss of function mutations.

A GAL1-counter selectable read-out system has been developed in the laboratories of ESBATech. We have applied this counter selection system in the context of SCINEX-P in order to extend its application for the screening of peptides or small molecules that display an inhibitory effect towards certain extracellular protein – protein ligations, or for loss of function mutations. Several yeast toxic markers have been used for negative selection, most notably the URA3 and CYH2. For negative selection both of these markers need addition of compounds that are either toxic (CYH2) or that are metabolised into a toxic substance by the reporter gene product (URA3). In small-molecule screenings however, it is not desirable to have reactive substances in the screening medium that might interfere with library molecules. For this reason, we employed genetic components involved in yeast
galactose metabolism to establish a counter selectable read-out in the SCINEX-P system.

The *GAL2* gene encodes galactose permease, which imports galactose into the cell. Intracellular galactose is a substrate of galactokinase (Gal1p), which converts galactose to galactose-1-phosphate (gal-1-P). In wild type cells Gal7p and Gal10p are involved in further metabolisation of galactose-1-phosphate into glucose-1-phosphate. In *GAL7* knockout cells gal-1-P metabolisation is abolished and gal-1-P accumulates to levels that are toxic to yeast cells. Such cells not only cannot use galactose as a carbon source, but also are unable to grow in presence of other sugars added to galactose because of accumulation of toxic gal-1-P. Genes involved in galactose metabolisation (gal-operon) in yeast are controlled by glucose- and galactose-dependent transcriptional regulators. Gal4p is a galactose dependent transcriptional activator controlling expression of the *GAL* genes. By knocking out the *GAL4* gene, its endogenous target genes *GAL1*, *GAL7* and *GAL10* are no longer expressed and consequently functionally knocked out as well. In *Δgal4* cells the *GAL1* open reading frame can thus be used to generate a reporter gene for counter selection on galactose containing media, since toxic galactose-1-phosphate accumulates in these cells upon expression of Gal1p (Gunde et al manuscript in preparation).

We stably integrated a synthetic bi-directional promoter containing the Hac1p binding sequence (UPRE) controlling the divergently oriented reporter genes *lacZ* and *GAL1*, at the *URA3* locus of a *GAL4* knockout strain (JPY-9). In this reporter strain, interactions of proteins fused to Ire1p C-terminal domains, through the UPR signalling cascade, would induce *lacZ/GAL1* reporter gene expression (Fig 1B) and result in growth inhibition on galactose. Two resulting reporter strain clones JUGal1-1 and JUGal1-2 were then transformed with empty vector or pDU44, an *ARS/CEN* plasmid expressing a constitutively active JunLZ-Ire1p fusion protein from the *ACT1* gene promoter. Transformants were plated on glucose and on galactose plates. JunLZ dimerisation caused activation of the Ire1p C-terminal domains and resulted in expression of the UPRE-*lacZ/GAL1* reporter genes. As depicted in Figure 1C, UPRE-*lacZ* expression levels of the two clones were inversely proportional to their ability to grow on plates containing galactose. High levels of reporter gene expression in JUGal1-1 even resulted in a complete deficiency to grow on galactose. Thus, this
system represents a means to screen for inhibitors of extracellular protein – protein interactions and may be amenable to high-throughput screenings of small molecule libraries on robotics platforms by using growth in liquid medium as read-out.

3.3.2. A second pathway to sense accumulation of unfolded proteins in the ER

Expression of soluble ligands

We have shown that extracellular protein interactions can be detected in a two-hybrid approach using the SCINEX-P technology \(^7\) (and chapter 3.3. of this thesis). Nevertheless, in a two-hybrid set-up the proteins of interest are forced into a certain orientation by the C-terminally-fused transmembrane portion of Ire1p, which might be disadvantageous for the detection of interactions comprising soluble factors such as ligands. Many hormone proteins, for example, bind in a multimer conformation to their cognate receptors. Multimer formation of some ligands may be hindered by the conformational constraints of a two-hybrid set-up. In contrast, co-expression of receptor chains fused to Ire1p derivatives together with soluble ligands directed to the secretory pathway would theoretically allow detection of receptor chain clustering upon ligand binding in the SCINEX-P system (Fig 2). Moreover, exclusive detection of interactions in the ER, and not on the cell surface, would restrict the resulting growth advantage to cells expressing a cognate ligand, thus making the system adequate for genetic screenings to identify novel soluble factors. We therefore attempted to use SCINEX-P to study interactions of the soluble murine ligands mEGF and mVEGF with their receptors mEGFR and mFLT1, respectively.

Soluble ligands in SCINEX-P induce UPR signalling in an unspecific manner.

The extracellular domains of mEGFR and mFLT1 were fused between the N-terminal signal sequence of the SUC2 gene (S2ss) and a wild type C-terminal portion of Ire1p extending from amino acid residue 495 to 1115. Ligands were directed to the ER by fusing the sequences coding for the mature proteins between S2ss and an ER retention signal (HDEL). These constructs were expressed from ARS/CEN plasmids under the
control of the \textit{ACT1} gene promoter in the UPR reporter strain IKU1-3, as shown in Figure 2A. Expression of ligands alone did not result in a detectable reporter gene activity. The mFLT1-Ire1p fusion weakly induced UPRE-\textit{lacZ} reporter gene activation, whereas mEGFR fusions strongly induced UPR signalling on its own. This may be in line with the finding that EGFR exists as a homodimer in the membrane already prior to ligand binding \cite{8}. A certain background activity of receptors fused to wild type Ire1p domains was expected since rare events of random receptor chain conjunctions are likely to occur in the two-dimensional space of the ER membrane.

UPR signalling activity of both receptors was further inducible by co-expression of ligands. But unexpectedly both ligands enhanced the ability of both receptor constructs to induce \textit{lacZ} expression. mEGF as well as mVEGF induced UPR signalling activity if co-expressed with either the mEGFR- or the mFLT1-Ire1p fusion to the same extend in a receptor type unspecific manner (Fig 2A). One plausible explanation is that over-expression of the receptor constructs might result in unselective aggregates of receptor and ligands, thus inducing UPR signalling. In order to lower expression level and background activity, the wild type Ire1p moiety in the mEGFR constructs was exchanged by either one of the two complementing Ire1p derivatives Ire1K702R\textsuperscript{495-1115} and Ire1\textsuperscript{Δ}tail\textsuperscript{495-982}. These new constructs were expressed from a very weak, galactose-inducible promoter containing only the fourth UAS\textsubscript{G} from the \textit{GAL1/10} promoter. Although background-signalling activity of mEGFR was strongly reduced with these constructs, both ligands still activated reporter gene expression unspecifically. mVEGF showed even a stronger potential to induce mEGFR dimerization than did mEGF (Fig 2B). Kaufman et al. \cite{9} found that replacement of the Ire1p lumenal domain by the c-Jun leucine zipper (JunLZ) constitutively activated UPR signalling. These constitutively active Ire1p derivatives were, however, still inducible upon addition of the UPR inducing agent tunicamycin, indicating that not only the Ire1p lumenal domain senses accumulation of unfolded proteins. It might therefore be that the mammalian ligands mEGF and mVEGF accumulate in the ER lumen as unfolded proteins and induce UPR signalling through the residual Ire1p moieties comprising its cytoplasmic part and its transmembrane domain.

In order to elucidate whether the observations described above are artifacts of soluble ligand expression and whether the respective receptors and ligands are
functional in yeast, we expressed receptor – ligand pairs in the membrane two-hybrid approach of SCINEX-P (Fig 2C).

Both mEGF and mVEGF bind to their receptors as homo-dimers. We thus excluded UPR induction due to ligand dimerisation by fusing the ligands between S2ss and the C-terminally deleted Ire1\textsuperscript{Δtail\textsubscript{495-982}} that does not exhibit residual signalling activity upon homo-dimerisation. The extracellular domains of the receptors were fused between S2ss and the kinase mutant Ire1\textsuperscript{K702R\textsubscript{495-111}}. These constructs were expressed from \textit{ARS/CEN} plasmid driven by the \textit{ACT1} gene promoter except for the mEGFR-Ire1p fusion, which was expressed from the weak, truncated \textit{ADH1} gene promoter, in order to lower the high background activity of this construct in SCINEX-P. In this setting both ligands, mEGF and mVEGF showed receptor specific reporter gene activation. As an additional control, the totally unrelated extracellular domain of Ost1p fused to Ire1\textsuperscript{Δtail\textsubscript{495-982}} did not induce UPR signalling above background levels if co-expressed with both receptor constructs (Fig 2C).

These data clearly show that cell surface receptors and their ligands can be functionally expressed in yeast when expressed as membrane attached fusion proteins in a two-hybrid set-up. In contrast, soluble expression of mEGF and mVEGF in the ER may induce unfolded protein response through the residual Ire1p portion in a receptor-independent manner.

\textit{Sensing accumulation of unfolded proteins in the ER lumen in the absence of transmembrane Ire1p.}

In order to test the hypothesis outlined above, namely that over-expression of ligands in the yeast ER might induce unfolded protein response, mVEGF and its receptor mFLT1 were examined for their potential to induce UPR signalling in an \textit{IRE1} wild type strain. While the receptor construct and mVEGF fused to Ire1\textsuperscript{Δtail\textsubscript{495-982}} did not significantly induce UPR signalling above the activities reached in a \textit{Δire1} strain, soluble mVEGF indeed activated UPRE-\textit{lacZ} reporter gene expression to almost 30\% of the value reached by co-expression of receptor and ligand in the same strain (data not shown). Expression of soluble mVEGF may thus induce unfolded protein stress in the yeast ER, which, through Ire1p derivatives, is signalled to the
nucleus and may thus be the cause for unspecific reporter gene activity previously reported.

To confirm this assumption we expressed three constructs containing the JunLZ as a dimerisation domain followed by different portions of wild type Ire1p (as depicted in Figure 4A) that resulted in i) a transmembrane protein (S-JunLZ-Ire1<sup>495-1115</sup>), ii) a fusion protein anchored in the membrane by a myristoyl residue attached to an N-terminal myristoylation box (M) (M-JunLZ-Ire1ΔTM<sup>555-1115</sup>) or iii) solubly expressed in the cytosol (JunLZ-Ire1ΔTM<sup>555-1115</sup>). Localisation of the two cytoplasmic constructs by fluorescence microscopy showed that M-JunLZ-Ire1ΔTM<sup>555-1115</sup> clearly localises to the plasma membrane, whereas JunLZ-Ire1ΔTM<sup>555-1115</sup> showed a dotted pattern all over the cell (Fig 3A), which we could not assigne to a certain cell compartment (Fig 3B). Most importantly both cytoplasmic Ire1p fusion proteins did not specifically stain the ER indicating that they are most probably uncoupled from the ER lumen. All three constructs, which are expected to constitutively form dimers due to the JunLZ moiety, spontaneously activated UPRE-lacZ reporter gene expression. Surprisingly, all of them where further inducible by tunicamycin treatment by about a factor 1.5 – 2 (Fig 4B). Tunicamycin inhibits N-linked glycosylation of secretory proteins and thus causes accumulation of unfolded proteins in the ER, which is sensed in the wild type yeast by full-length Ire1p.

Induction of UPR signalling by tunicamycin in the presence of the transmembrane Ire1p derivative could be explained by the residual 30 amino acids of the Ire1p lumenal domain, which might still sense accumulation of unfolded proteins. The two cytoplasmic Ire1ΔTM constructs, in contrast, cannot directly communicate with the ER lumen. Signal transduction from the ER lumen to the cytosol must therefore occur via an alternative pathway. In an attempt to define the step in which a putative second UPR pathway discharges into the Ire1p/Hac1p signalling cascade, we ectopically expressed a spliced version of the HAC1 open reading frame from an ARS/CEN plasmid under the control of the ACT1 promoter in Δhac1 cells. Constitutive expression of Hac1p resulted in constitutive UPR signalling, which was only slightly, if at all, inducible by tunicamycin treatment (Fig 4C). Co-expression of S-JunLZ-Ire1<sup>495-1115</sup>, the constitutively active transmembrane Ire1p construct, showed no effect in this Δhac1 strain indicating that active Ire1p does not influence Hac1p activity at the protein level.
Our observations that a) constitutively active cytosolic Ire1p fusions are further inducible by generation of stressful conditions in the ER lumen and b) that the reporter gene activity upon constitutive expression of Hac1p is not significantly inducible by such conditions, suggest an enhancement of processes between Ire1p activation and Hac1p translation by a redundant pathway transmitting a signal from the ER lumen to the cytosol. The activity of the ACT1 gene promoter did not significantly increase upon tunicamycin treatment as determined by measuring β-galactosidase expression from a PACT1-lacZ vector, excluding the possibility that simply expression of the Ire1p-fusion proteins was enhanced by tunicamycin (data not shown). Already in 1996 Shamu and Walter found that about one-third of the KAR2 promoter activation is independent of IRE1. A genome-wide analysis identified 87 open reading frames (ORF) that are induced in response to ER stress in Δire1Δhac1 strains. Recently, Schroder et al. found that the mediator complex member Sin4p is involved in IRE1/HAC1 independent UPR signalling. ER-stress inducing agents again activated UPRE-lacZ reporter gene expression in Δsin4Δire1Δhac1 cells, in contrast to Δire1Δhac1 cells. Moreover, activation of transcription was independent of UPREs in the reporter gene promoter. The authors called this effect core promoter activation (CPA), which was specific for a certain subset of genes whereof most were UPR targets. Schroder et al. claim that the very weak basal expression of UPR target genes is the reason why activation of these genes by UPR inducing agents was not detectable in Δhac1Δire1 SIN4 wild type cells. According to one model, SIN4 deletion increases basal transcription of these UPR target genes, and only upon increasing basal activity the effect on expression by an alternative UPR pathway is detectable. CPA contributed about 10% to the activation of the UPR target gene PDI1 and to even higher extend to induction of the UPRE-lacZ used in the Schroder study, which consisted of the CYC1 core promoter (an UPR target gene) containing an inserted UPRE. Interestingly, not every UPR target gene was inducible by CPA. SCJ1 and EUG1 for example are not targets of core promoter activation upon ER stress.

The UPRE-lacZ reporter construct used in our study consisted of the GAL1 core promoter containing an inserted UPRE. GAL1 expression was not induced by tunicamycin neither in wild type nor in Δire1Δhac1 cells (Travers et al.) and is thus not a target of UPR or CPA. Consequently, CPA is rather unlikely to explain a 1.5 – 2 fold activation of our UPRE-lacZ reporter gene by tunicamycin, which we observed.
in Δire1 cells expressing a constitutively active cytoplasmic Ire1p-construct. Furthermore, if HAC1/IRE1 independent pathways were responsible for additional transcriptional activity, we would expect equally strong core promoter activation independent of which factor in the UPR cascade is constitutively active. This expectation is in contrast to our findings that only the signal obtained from constitutive active Ire1p and not from Hac1p was further inducible. Our current data, however, cannot exclude that over-expression of Hac1p in our experiments overweighs the effect of an alternative pathway that activates reporter gene expression. Integration of the HAC1i open reading frame (ORF) into the HAC1 locus might help excluding artifacts resulting from over-expression. An alternative approach to investigate the endpoint of a putative second pathway is to determine the ratio of HAC1i mRNA versus HAC1u mRNA by RNA mapping analysis, in cells expressing constitutively active cytoplasmic Ire1p constructs in the presence and absence of tunicamycin. Assuming that the second UPR pathway activates transcription in an IRE1/HAC1 independent mechanism, HAC1u mRNA splicing should not be induced by tunicamycin in this set-up.

3.3.3. Conclusions

Expression of soluble ligands into the secretory pathway seems to induce UPRE-reporter gene expression through an unknown pathway and moreover in a receptor independent manner. Our results obtained with the cytoplasmic Ire1p constructs indicate that a putative second pathway discharges into the known UPR cascade somewhere between Ire1p activation and Hac1p translation. The findings of Schroder et al. in contrast suggest a HAC1/IRE1 independent signalling that activates UPR target genes in a UPRE independent manner. Identification of members of this pathway would in any case provide information for a rational approach to circumvent an unspecified UPR signalling in order to afford detection of interactions between soluble ligands and their cell-surface receptors.

Since the UPR target genes SCJ1 and EUG1 are not inducible by CPA their promoters could be utilised in reporter constructs instead of the artificial promoter used in this study, consisting of the GAL1/10 core promoter, containing inserted
UPRE sequences. Application of *SCJ1* and *EUG1* promoters in SCINEX-P would possibly uncouple UPR from CPA and allow screenings for soluble ligands in Δire1 cells.
3. 3. 4. Materials and methods

Recombinant plasmids construction

Expression vectors: extracellular domains of mouse EGFR, mFLT1 and the ligands mEGF and mVEGF where amplified by PCR from a mouse cDNA library. The PCR primers binding to the 5’ of the respective sequence contained an XbaI site followed by the SUC2 signal sequence (S2ss) flanking the homology region. The S2ss substituted for the mammalian secretion signal of the respective genes. All primers binding to the 3’ sequence of the gene of interest (GOI) used to generate fusions to the Ire1p C-termini, were flanked by a NotI restriction site allowing in-frame fusion to the Ire1p C-termini resulting in the junction GOI-\textbf{ggc ggc ege-IRE1} (NotI site bold). 3’ primers used to generate soluble ligands contained the sequence coding for the ER retention signal (HDEL) followed by a NotI site flanking the homology region. The JunLZ fragments containing an N-terminal S2ss and a C-terminal HA tag were amplified by PCR using pDU44 as template and a 3’ primer providing the HA tag and a flanking NotI site. Ire1 C-termini were amplified by PCR as described in 7. The genes of interest and IRE1 parts where cloned into the XbaI/Sall treated yeast expression vectors pDU10 (PACT1, ARS/CEN, LEU2), pDU12 (PACT1, ARS/CEN, TRP1) or pMH4 (PADH1, ARS/CEN, LEU2). Construction of these vectors as well as of pDU44 (PACT1, ARS/CEN, TRP1) expressing S-JunLZ-Ire1\textsuperscript{555-1115} was described elsewhere 7. The GAL1/10 promoter, deleted for UAS\textsubscript{G} 1-3 was constructed by PCR amplification using a 5’ primer of the sequence 5’-\texttt{gaattccgtcctcgtcctcaccggtc}-3’ annealing upstream of UAS\textsubscript{G} 4 and containing a flanking EcoRI site, and a 3’ primer of the sequence 5’-\texttt{gcttactgtagttttttttcttcggg}-3’ annealing downstream of the TATA box and containing a flanking XbaI site. The resulting PCR product was cloned into EcoRI/XbaI treated pDU10.

Integration vectors: The UPRE-Gal1-lacZ reporter plasmid pDU101 was constructed by substituting the six LexA binding sites in the bi-directional GAL1/10 promoter of pDE94-Gal1 by one UPRE from pPL1 by the use of unique BamHI and SalI restriction sites flanking the \textit{cis}-activating sequences in both plasmids.
Yeast strains & media

The *S. cerevisiae* strain JUGal1 was generated by transformation of JPY-9 (*ura3-52; his3Δ200; leu2Δ1; trp1Δ63; lys2Δ385; gal4Δ11; MATα*) with the reporter construct pDU101 carrying the divergently oriented *lacZ* and *GAL1* reporter genes under the control of a bi-directional promoter containing one UPRE from the *KAR2* promoter. The plasmid was linearised prior to transformation at the *BstBI* site in the *URA3* gene. Growth on uracil lacking plates selected for stable integration in the *URA3* locus, which was confirmed by PCR analysis. Generation of the *s. cerevisiae* strain IKU1-3 was described elsewhere. *HAC1* knockouts were done in the UPRE reporter strain YPL-2 by replacing the *HAC1 ORF* by the selectable marker *kanMX4*. Yeast genetics and media were as described. For selection of plasmids, dropout media containing all except the specified amino acid were used. Yeast transformation was performed by the lithium acetate procedure. Tunicamycin was added to a final concentration of 1mg ml\(^{-1}\) to liquid cultures 3 hours prior to determination of β-galactosidase activity. Counter selection on plates: JUGal1 clones were transformed with indicated plasmids and cells were plated on selective plates containing 2% glucose or 3% galactose, 2% raffinose and 2% glycerol instead of glucose. Cultures were incubated at 30°C.

Assay of cellular β-Galactosidase activity

β-Galactosidase activity of cells was determined using the protocol described in. Activity was normalised to the number of cells assayed.

Immunostainings

The protocol used for immunolocalisation of cytoplasmic Ire1p fusion proteins was performed as described. The monoclonal mouse anti-HA antibody (SIGMA) was used as a primary antibody in a working dilution of 1:50 in PEMBAL. The secondary antibody against mouse IgG was coupled to the fluorophor Cy3 (Jackson
ImmunoResearch Laboratories Inc.) and diluted 1:500 in PEMBAL. Fluorescence was observed with a 100-x objective on a Leica microscope (Leica DM R). Exposure parameters were adjusted to signal strength. For staining of nuclear DNA with DAPI (4,6-diamidino-2-phenylindol), the fixed, spheroplasted, and permeabilised yeast cells were incubated with 1 microgram/ml DAPI in 100% ethanol for 1 minute followed by extensive washing with 1xPBS.
3.3.5. Figures and Figure legends

Figure 1. The GAL1 counter selectable read-out in the SCINEX-P system. A) The GAL2 gene product promotes internalisation of extracellular galactose (gal). Inside the cell galactose is converted to galactose-1-phosphate (gal-1-P) by the GAL1 gene product the galactokinase. The GAL7 gene product (uridylkinase) catalyses production of glucose-1-Phosphate (glu-1-P) and UDP-galactose. UDP-galactose is recycled to UDP-glucose (UDP-glu) by the GAL10 gene product (in grey). Mutants lacking GAL7 activity accumulate gal-1-phosphate, which in high levels is toxic to yeast cells. B) Homo-dimer formation of c-Jun leucine zippers (JunLZ) brings the C-terminally fused Ire1p moieties into close proximity allowing auto-phosphorylation and subsequently, through Hac1p, activate transcription of the UPRE-GAL1-lacZ reporter genes. In presence of galactose, expression of the GAL1 gene product leads to accumulation of toxic gal-1-P, which inhibits growth. C) Counter selection on galactose: JUGal1-1 and JUGal1-2 cells were transformed with either a vector expressing the constitutively active Ire1p fusion protein (S-JunLZ-Ire1p) or an empty vector. JUGal1-1 cells strongly express the UPRE-GAL1-lacZ reporter genes, as determined by measuring β-galactosidase activity, and do thus not grow on glucose containing medium. JUGal1-2 cells express the reporter genes to a lower extend than JUGal1-1 cells and consequently exhibit a milder phenotype on galactose.
Figure 1

A

B

C

Glucose  Galactose

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Figure 2. Co-expression of receptors and ligands in SCINEX-P. UPR signalling activity was quantified by measuring β-galactosidase activity in transformed IKU1-3 cells in liquid cultures. A) Co-expression of receptor extracellular domains (R_{ECD}) of mEGFR and mFLT1 with their solubly expressed ligands mEGF and mVEGF, respectively. All constructs were expressed from the ACT1 gene promoter (P_{ACT1}). Receptors were fused to wild type C-terminal domains of Ire1p (Ire1p_{526-1115}). B) R_{ECD} of mEGFR was expressed as fusion to either one of the two complementing mutant derivatives of Ire1p (Ire1K702R_{526-1116}) and Ire1Δtail_{526-982}) under the control of the truncated GAL1/10 promoter (P_{GAL1(4)}) in galactose containing medium. C) Expression of R_{ECD} of mEGFR and mFLT1 together mEGF, mVEGF and the extracellular domain of Ost1 in the two-hybrid set-up of SCINEX-P. mEGFR was expressed from the truncated ADH1 promoter (P_{ADH1}).
Figure

A

Ire1wt

Ricc

β-Galactosidase units

PGAL1-mEGFR-Ire1tail

B

Ire1Δtail

Ricc

PGAL1(4)-mEGFR-Ire1Δtail

C

Ire1K702R

Ricc

PGAL1(4)-mEGFR-Ire1K702R

β-Galactosidase units

PGAL1(4)-mEGFR-Ire1tail

PACT1-mFLT1-Ire1tail

PACT1-mEGFR-Ire1tail

PACT1-mEGF-HDEL

PACT1-mVEGF-HDEL

PACT1-mFLT1-Ire1K702R

PACT1-mEGFR-Ire1K702R

PACT1-mEGF-HDEL

PACT1-mVEGF-HDEL

PACT1-mVEGF-Ire1Δtail

PACT1-Ost1-Ire1Δtail
**Figure 3. Immunostaining of cytoplasmic Ire1p fusion proteins.** IKU1-3 cells were transformed with the plasmids expressing M-JunLZ-Ire1ΔTM^{555-1115} or JunLZ-Ire1ΔTM^{555-1115} or with an empty vector. **A**) Localisation of the fusion proteins was determined by fluorescence microscopy using a primary antibody directed against the HA-tag in the respective constructs. **B**) Staining of JunLZ-Ire1ΔTM^{555-1115} using the anti HA antibody, and DAPI staining of the nucleus.
Figure 3

A

anti-HA

NOMARBG | FLUORESCENCE

mock

M-JunLZ-Ire1ATM^{665-1115}

JunLZ-Ire1ATM^{665-1115}

B

anti-HA | DAPI | OVERLAY

JunLZ-Ire1ATM^{665-1115}
Figure 4. Sensing accumulation of unfolded proteins through an IRE1-independent mechanism. A) Schematic drawing of the Ire1p fusion proteins expressed in IKU1-3 cells (shown in B and C). SUC2 signal sequence (S2ss), myristoylation box (M), leucine zipper of c-Jun (JunLZ), mammalian hemagglutinin tag (HA), C-terminal domain of Ire1p extending from amino acid 495 to 1115 (Ire1p\(^{495-1115}\)), C-terminal domain of Ire1p lacking its transmembrane domain and extending from residue 555 to 1115 (Ire1\(\Delta\)TM\(^{555-1115}\)). All constructs were expressed from the \(ACT1\) gene promoter. B) β-galactosidase reporter gene activity obtained upon expression of constitutively active Ire1p fusion proteins in the \(\Delta ire1\) strain IKU1-3 before and 3 hours after addition of 1mg ml\(^{-1}\) tunicamycin to the medium. C) β-galactosidase reporter gene activity upon expression of the spliced \(HAC1\) ORF under the control of the \(ACT1\) promoter with and without co-expression of a constitutively active Ire1p fusion protein in a \(\Delta hac1\) UPRE reporter strain. Signals were measured before and 3 hours after addition of 1mg ml\(^{-1}\) tunicamycin to the medium.
Figure 4

A

B

C

Δire1

Δhac1
3.3.6. References


4. GENERAL CONCLUSIONS

Interactions among extracellular and transmembrane proteins regulate a variety of important physiological processes and are involved in a multitude of diseases. A detailed understanding of the interaction network among extracellular and cell surface proteins would allow us to gain insight into complex physiological processes and would probably facilitate rational approaches to therapy. Recent attempts to map the entire interactome of a given organism by use of the classical yeast two-hybrid method have shown that this method is biased to intracellular protein interactions (reviewed by 1). Of the 8988 known human genes in the ENSEMBL database containing a SWISSPROT ID, 2331 (25.9%) contain a signal sequence, and 2391 (26.6%) contain a transmembrane domain. Taking in account that only 14.1% bear both, a signal sequence as well as a transmembrane domain leads to the assumption that almost 40% of the known genes in the ENSEMBL database code for secreted or transmembrane proteins. Equal numbers were obtained by screening other human protein databases for these two features. It remains yet unclear whether these data are representative for the entire human genome. Many of the putatively extracellular/transmembrane proteins are still not assigned to a certain function. For example in a recently published high throughput attempt to identify novel human secreted and transmembrane proteins, in which bio-informatic and genetic methods have been combined, a total of 1047 cDNA clones have been identified, 256 of which were novel genes of unknown function 2. Though, the identification of binding partners of secreted and transmembrane proteins is complicated by their biochemical properties.

The problems arising with transmembrane proteins regarding attempts to identify and characterise interaction partners can be exemplified by G protein coupled receptors (GPCRs), which represent a pharmacologically important class of transmembrane proteins. At present 39 of the top 100 marketed drugs in use directly or indirectly act through activation or blockade of GPCR-mediated receptors. Nearly 160 GPCRs have been identified based on their gene sequence and their ability to interact with known endogenous ligands. However, an estimated 500-800 additional GPCRs have been classified as "orphan" receptors (oGPCRs) because their endogenous ligands have not yet been identified 3. Ligands that bind to disease
relevant oGPCRs or agents that block the interactions between a certain ligand and its receptor may be used for therapy. GPCRs are difficult to purify in high amounts and to keep functional after solubilisation and purification. Thus biochemical methods for the identification of peptides or molecules that interact with GPCRs in high throughput approaches are rather inappropriate. For this class of proteins also conventional genetic systems to detect protein interactions, such as nuclear or cytoplasmic yeast two-hybrid methods are inapplicable since transmembrane proteins are unlikely to fold properly if expressed in the cytoplasm, and their hydrophobic transmembrane domains tend to aggregate in an aqueous environment. It exists therefore a great need for genetic systems that are compatible with the requirements of extracellular and transmembrane proteins, and which are amenable for large-scale screenings. However, among the genetic systems able to detect interactions between membrane-attached proteins (such as DHFR complementation, β-galactosidase complementation, G-protein fusions and the split-ubiquitin system as reviewed by Stagljar and Fields 1), to our knowledge none has been successfully used to date for the identification of extracellular interaction partners. Although we did not demonstrate the feasibility of SCINEX-P for multispansing transmembrane proteins, we believe that extracellular interactions comprising GPCRs can be reconstituted in this system. This assumption is supported by the fact that the mammalian oGPCR FPRL-1 was functional when expressed as a fusion protein in yeast 4.

4.1. Monitoring extracellular protein interactions in SCINEX-P

In part 3.1. of this work we demonstrate the feasibility of SCINEX-P to detect protein interactions occurring in the topologically extracellular compartment of the ER. We used the leucine zipper of GCN4 (GCN4LZ), which contains dimerization activity, as an epitope, and various single-chain antibody fragments (scFvs) to demonstrate the ability of the SCINEX-P system to specifically detect protein – protein interactions in the lumen of the ER. In this set-up the epitope was expressed as a fusion to Ire1Δtail in order to prevent UPR induction due to GCN4LZ homo-dimerization. Antibody fragments where fused to Ire1K702R. Co-expression of the epitope construct together with the GCN4LZ specific scFvs λ-graft or α-GCN4wt resulted in reconstitution of
UPR signalling, while co-expression of the epitope with the non-binder AL-5 did not. In contrast to the very stable \( \lambda \)-graft \( \alpha \)-GCN4wt was not functional if expressed in the reducing environment of the cytosol \(^5\), where disulfide-bonds cannot form. In the SCINEX-P system, however, reporter gene activity obtained by co-expressing the GCN4LZ epitope with \( \alpha \)-GCN4wt was as high as that observed with the even intracellularly stable \( \lambda \)-graft, indicating that extracellular proteins are properly folded in SCINEX-P. Mutating the cysteine residues in \( \alpha \)-GCN4wt (\( \alpha \)-GCN4(SS--)) resulted in a loss of \textit{in vivo} binding activity in SCINEX-P. \( \alpha \)-GCN4(SS--) did no longer induce UPR signalling above background level, thus underlining the importance of disulfide bond formation for functionality. UPRE-\textit{lacZ} reporter gene activity correlated with growth on selective plates, thus \( \Delta \ire \) cells expressing a pair of interacting extracellular proteins could be selected by growth on media challenging different read-out systems (inositol depletion, histidine depletion and exposure to elevated temperature). By assessing combinations of different selective pressures the stringency of growth selection could be modified. In addition, we showed that the Ire1p cytoplasmic portion, lacking both the N-terminal lumenal domain as well as the transmembrane domain, is sufficient for UPR induction and can thus be fused to the C-term of full-length transmembrane proteins for identifying extracellular and intracellular interaction partners.

**4.2. Screening for extracellular protein interactions in SCINEX-P**

In 3.2, the SCINEX-P system was then applied to screen a scFv fragment library for binders to the cytokine interleukin-13 (IL-13). Both, IL-13 as well as scFv fragments are extracellular proteins containing disulfide-bonds, which are essential for proper folding. The scFv library was based on a framework of the \( \lambda \)-3-VH1 type containing seven randomised amino acid residues in the complementarity-determining region 3 of the heavy chain. Binders obtained in a primary screening where randomised in four amino acid residues of their complementarity-determining region 3 in the light chain by homologous recombination in yeast and subjected to a second screening under higher selective conditions. Positives from this second affinity maturation screening with improved \textit{in vivo} binding activity were purified and further analysed \textit{in vitro}. The
results obtained from Western blot experiments and from ELISA indicate that the scFv fragments deriving from the affinity maturation screening indeed exhibit a higher affinity to their epitope than the original binders resulting from the primary screening. Thus growth behaviour and reporter gene expression were in good correlation with in vitro results, demonstrating the feasibility of SCINEX-P for genomic screenings to identify extracellular protein – protein interactions. We could, however, not reproduce the interactions between hIL-13 and the scFv fragments obtained from the SCINEX-P screenings in a classical nuclear yeast two-hybrid approach (data not shown). This indicates that either hIL-13 or the scFv fragments (or both) are not functional if expressed in the cytoplasm, underlining the importance of extracellular protein interactions to be reconstituted in a cognate environment.

4.3. Outlook

In both the primary screening and the affinity maturation screening we encountered a relatively high number of false positives, 84% and 90%, respectively, which activated the UPRE-lacZ reporter gene in a bait-independent manner. The presence of scFv fragments possessing homo-dimerisation activity, which consequently activate the C-terminally fused kinase mutant derivative of Ire1p (Ire1K702R), is the most probable explanation for bait-independent UPR signalling. Such bait-independent clones could readily be sorted out, since they did no longer stain blue on X-Gal plates upon loss of the URA3-bait-plasmid on 5FOA plates. Nevertheless, in cDNA screenings, clones exhibiting dimerisation activity are not necessarily false positives, since they might still interact with the bait protein. It would therefore be more convenient to exclude false positives that do not interact with the bait from the beginning. This could be achieved by expressing the library as a fusion to the Ire1Δtail mutant instead of Ire1K702R, since Ire1Δtail contains no residual UPR signalling potential and thus, in contrast to Ire1K702R, cannot be activated by homo-dimer formation. This approach, however, assumes that a particular bait protein, fused to Ire1K702R does not form dimers on its own.

The ultimate solution to exclude false positives resulting from homo-dimerisation, however, would be to use a mutant Ire1p derivative that bears no
residual signalling potential, but whose activity could still be complemented by the tail deletion mutant Ire1Δtail. Such kinase mutants, though, have not yet been identified. SCINEX-P could be employed to identify such Ire1p derivatives by screening for loss of function mutants in the UPR-counter selectable reporter strain JUGal1 (described in 3. 3) in a first round, and, in a second round selecting those among the pool of inactive mutants that can reconstitute UPR signalling if co-expressed with the Ire1Δtail mutant. This second selection for the ability to complement Ire1Δtail could be performed in the UPR-positive selectable IKU3-3 strain.
4. References


5. APPENDIX

5.1. Genotypes of the strains

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5.2. Yeast plasmids

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Screening for extracellular protein interactions in yeast

**Integration vectors**

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M = myristoylation signal; S2ss = SUC2 signal sequence; ADH trunc. = truncated ADH1 gene promoter; ecd = extracellular domain.
5. 3. Acknowledgements

My deepest gratitude belongs to Professor Howard Riezman and Dr. Alcide Barberis. Howard Riezman accepted me as his Ph. D. student and continuously supported my projects. As a member of the scientific advisory board he generally contributed to ESBATech’s scientific progress. I thank Alcide Barberis for his enthusiasm and his valuable intellectual contributions that helped me bringing yeast to bite the dust on selective conditions. After some refreshing fights in the beginning of my time at ESBATech AG, he became a good friend whose sense of humour I appreciate very much. I also thank Dominik Escher, the CEO of ESBATech AG, for giving me the opportunity to realise my ideas and for his readiness to rig a Ph.D. students education program, offering us to participate at scientific conferences all over the world. Due to the generosity of Alcide and Dominik, the company’s needs and my scientific interests were combined in a fruitful compromise.

For their contribution to a joyful environment I thank:
Oli “the bottomless pit” Middendorp who always spread a corybantic athmosphere around him, Tea “the quiet water” Gunde who turned out to be much more bubbly than it seems, Urs “the second first Klugscheisser” Lüthi with whom I spent many hours above 3000m (and for whom one pepperoni and 1/6 of a “tessiner” bread is enough to eat during a 12 hours ascent from 800 to 3600m.a.s.), Stefan “early bird” Tanner, who tried to teach me biking, snowboarding and other funs, Karin “yes I’ll be here on Sunday and can inoculate your samples” Edler who listens to appalling music, Peter “colour code” Lichtlen who is my oldest companion at ESBATech, Kirsten “mutty” Mundt whose pretty face decorated ESBATech’s first homepage, and Valerie “relax” Cottier who I’ve never seen exited. Maya “twister” Furler, Catherine Berset and Julia Tiez and all the others belonging to the ESBATech family, also contributed to the constructive environment I met at ESBATech AG.

I am grateful to my girlfriend Rahel Brunner. Her job was to even the bipolarity of my mood that was strongly dependent on success and failure in the lab. With her adventurous habit, she fulfilled her duty with commendation.

Last but not least I would like to express my gratitude to my parents who supported me whenever there was a need to.
5. 4. Curriculum vitae

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Ausbildung

1981 – 1987 Primarschule, Meilen/ZH
1987 – 1990 Sekundarschule, Meilen/ZH
1990 – 1995 Kantonsschule Hottingen, Matura Typus E (Wirtschaft)
1996 – 2000 Studium der Biologie, Universität Zürich

Okt. 1997 1. Vordiplom
Okt. 1998 2. Vordiplom

Diplomarbeit: “The promoter region of the metal responsive transcription factor (MTF-1): repression by cis- and transactive components”
Unter der Aufsicht von Prof. Walter Schaffner
Institut für Molekularbiologie (IMB), Universität Zürich

2001 – 2004 Dissertation in der Biotechnologie-Firma ESBATech AG, Schlieren, unter der Leitung von Dr. Alcide Barberis (ESBATech AG) und Prof. Howard Riezman (Biozentrum, Basel)

Titel der Dissertation: “Screening for extracellular protein – protein interactions in a novel yeast growth selection system”
Veröffentlichungen


Meetings

“Yeast2003” Conference, Göteborg, Sweden, June 2003
Short oral presentation: *A growth selection system to detect extracellular and transmembrane protein interactions*

Keystone Symposium on Angiogenesis and Cancer, Banff, Canada, February 2002

Arolla Conference on Molecular Biology, Arolla, Switzerland, 2000

Present position: Ph.D. student with ESBA Tech AG, Schlieren