In vitro selection and prediction of TIP47 protein-interaction interfaces

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We present a new method for the rapid identification of amino acid residues that contribute to protein-protein interfaces. Tail-interacting protein of 47 kDa (TIP47) binds Rab9 GTPase and the cytoplasmic domains of mannose 6-phosphate receptors and is required for their transport from endosomes to the Golgi apparatus. Cysteine mutations were incorporated randomly into TIP47 by expression in *Escherichia coli* cells harboring specific misincorporator tRNAs. We made use of the ability of the native TIP47 protein to protect 48 cysteine probes from chemical modification by iodoacetamide as a means to obtain a surface map of TIP47, revealing the identity of surface-localized, hydrophobic residues that are likely to participate in protein-protein interactions. Direct mutation of predicted interface residues confirmed that the protein had altered binding affinity for the mannose 6-phosphate receptor. TIP47 mutants with enhanced or diminished affinities were also selected by affinity chromatography. These methods were validated in comparison with the protein's crystal structure, and provide a powerful means to predict protein-protein interaction interfaces.

Protein-protein interactions are of fundamental importance to all aspects of cell physiology. Yet conventional methods to analyze these interactions in detail require the presence of independent, previously characterized binding domains (such as SH2 domains) and/or the availability of the three-dimensional structures of the molecules involved. We describe here a new approach to identify surface-localized, hydrophobic residues that have the potential to contribute to protein-protein interaction interfaces. This technique is not limited by protein size, complexity or solubility and can be performed with microgram quantities of protein. The method makes use of a recent technique that reveals protein topology through the use of chemical modification[1]. In addition, we show here that high-throughput mutation of amino acid residues by translational misincorporation, combined with affinity chromatography, offers an additional, efficient method to select, *in vitro*, for mutants with enhanced or decreased binding-partner interaction. Together, these techniques can rapidly identify functional residues within protein-protein interaction interfaces in the context of most full-length proteins of interest.

TIP47 (tail interacting protein of 47 kDa) binds to the cytoplasmic domains of mannose 6-phosphate receptors (MPRs) and is required for their transport from endosomes to the Golgi[2–5]. When TIP47 is depleted from cells using antisense oligonucleotides, MPRs are mis-sorted to the lysosome and degraded[2]. TIP47 is predominantly cytoplasmic, and a fraction is recruited onto endosome membranes through interaction with MPRs and the Rab9 GTPase, which are localized there[2,5,6]. Upon binding to Rab9, TIP47 shows an enhanced affinity for MPR cytoplasmic domains, and a ternary complex is formed[5].

We have used misincorporation proton-alkyl exchange (MPAX)[1] to map amino acid residues that lie on the surface of native TIP47 and to predict that certain of these contribute partner protein binding. We report further the selection of two TIP47 proteins with enhanced or diminished ligand affinity *in vitro*. The TIP47 crystal structure[7] validated the usefulness of this approach for difficult-to-crystallize proteins. This use of MPAX provides a new means to identify amino acid residues critical for biological function.

RESULTS

Cysteine misincorporation in TIP47

We used MPAX[1] to examine the solvent accessibility of amino acid residues in native TIP47 protein. This technique makes use of low-frequency cysteine misincorporation that occurs when proteins are expressed in cells harboring specific mutant cysteine tRNAs. A synthetic TIP47 gene was generated that comprised codons complementary to the specific cysteine-misincorporator tRNAs used to optimize cysteine misincorporation. Note that this is not an essential step but will provide maximal misincorporation. TIP47 was coexpressed with misincorporator tRNAs specific for either valine, isoleucine, leucine, glutamine, tryptophan or phenylalanine, and then purified and radiolabeled. This method yields an ensemble of TIP47 proteins in which a single cysteine residue per protein molecule is incorporated at random in place of a given amino acid.

MPAX takes advantage of the fact that cysteine residues within a polypeptide chain can effect polypeptide cleavage upon cyanylation with 2-nitro-5-thiocyanobenzoic acid (NTCB) followed by incubation in ammonia[1,8,9]. Resulting cleavage products are then

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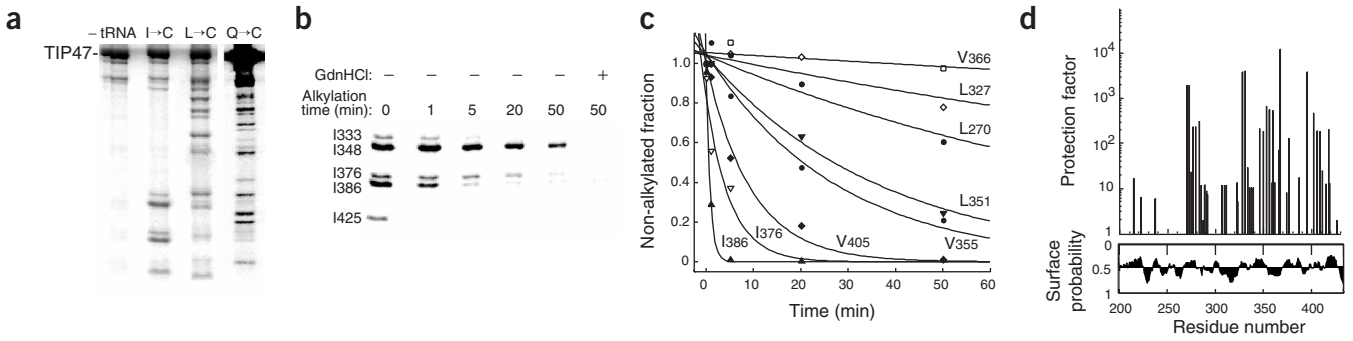

Figure 1 | Biochemical readout of cysteine misincorporation in TIP47. (**a**), Cysteine-free TIP47 was expressed in the absence (–tRNA) or presence of isoleucine (I→C) , leucine (L→C) or glutamine (Q→C) misincorporator tRNAs that introduce cysteine at a low frequency into TIP47. Radiolabeled TIP47 mutants were cleaved after cyanylation under denaturing conditions and products separated on tricine gels. The uncleaved protein is indicated. (**b**) Protection of cysteine probes by protein structure. TIP47 mutant pools were alkylated for the indicated times, either in the presence of guanidine hydrochloride (GdnHCl; lane 6) or in its absence. Proteins were then denatured, cyanylated and cleaved. (**c**) Kinetics of cysteine alkylation. TIP47 was alkylated for various times with 10 mM iodoacetamide and cleaved with 2-nitro-5-thiocyanobenzoic acid. Alkylation protection from cleavage, as a function of time of alkylation, is shown for cysteine substitutions at Val366, Leu327, Leu270, Leu351, Val355, Val405, Ile376 and Ile386. Data were fit to a first-order exponential decay curve. (**d**) Top, protection factor map of TIP47. Rate constants for 48 TIP47 cysteine substitutions were compared to the intrinsic rate constant of alkylation of Ile→Cys mutations in a fully denatured TIP47 peptide according to the relation:protection factor = $K_{intrinsic}/K_{observed}$. The protection factors for misincorporation at valine, leucine, isoleucine, phenylalanine and glutamine residues in TIP47 are shown. All rates were dependent upon iodoacetamide concentration, confirming that conformational closing was faster than the intrinsic alkylation rate. Below, a prediction of the regions of TIP47 most likely to be surface localized[14].

resolved using a tricine gel. When TIP47 proteins containing misincorporated cysteine residues were denatured with guanidine HCl and then cleaved, distinct and characteristic cleavage patterns were detected (**Fig. 1a**). Proteins from bacteria expressing distinct tRNAs generated unique patterns, in which each band corresponded to a particular residue in the TIP47 sequence (**Fig. 1a**, lanes 2–4). As expected, when TIP47 purified from cells lacking a misincorporator tRNA was treated in parallel, no products were obtained (**Fig. 1a**, left lane). The minor doublet seen below TIP47 in lane 1 appears to be a degradation product that did not interfere with our subsequent analyses.

Solvent accessibility of TIP47 residues

The chemical reactivity of cysteine residues with the alkylating agent iodoacetamide provides a measure of residue burial in triosephosphate isomerase, a protein that forms a $(\beta/\alpha)_8$ barrel structure[1]; buried residues are alkylated more slowly than those present on the protein surface. We used TIP47 containing randomly misincorporated cysteine to probe the solvent accessibility of these residues across the polypeptide sequence.

The kinetics of iodoacetamide alkylation at different positions in TIP47 was monitored for native TIP47 using alkylation in conjunction with NTCB cleavage. Solvent-exposed thiols that are alkylated resist modification by NTCB and are not subject to chemical cleavage; buried thiols are protected from alkylation by protein structure, and can be revealed by subsequent denaturation and NTCB-driven cleavage.

When TIP47 molecules containing roughly one cysteine residue in place of a random isoleucine residue were first unfolded with guanidine HCl and then incubated for 50 min with iodoacetamide, all cysteine residues became alkylated. In this situation, no sites would be available for NTCB modification; as expected, no cleavage products were observed (**Fig. 1b**, lane 6). In contrast, when native TIP47 was incubated in the absence of iodoacetamide and then unfolded, cysteines at all possible positions could be cyanylated by NTCB and the protein backbone was cleaved (**Fig. 1b**, lane 1). Five cleavage products were obtained corresponding to cleavage at positions normally occupied by isoleucines 333, 348, 376, 386 and 425. This protocol was then used to map the relative alkylation rates of misincorporated cysteine residues in TIP47. Proteins were treated for various times with iodoacetamide, after which they were unfolded and subjected to cleavage. Cleavage products disappeared with time of alkylation; the rates of their disappearance reflected

Table 1 | Half-life of free cysteine thiols in TIP47 during alkylation

Residue	Half-life (min)	Residue	Half-life (min)
Ile214	0.8	Gln336	0.5
Phe221	0.3	Gln338	0.5
Phe236	0.3	Leu344	10.3
Leu269*	94.5	Ile348	9.3
Leu270*	94.5	Leu351	34.1
Leu272*	94.5	Val355	27.5
Gln274	1.1	Gln358	0.7
Leu276*	11.5	Val359	27.1
Leu278*	11.5	Gln360	0.7
Val282	15.8	Gln361	0.7
Gln284	0.6	Gln365	3.6
Val286	0.1	Val366	618.4
Gln288	0.6	Phe373	0.4
Leu290	0.4	Ile376	6.6
Val291	0.4	Ile386	0.9
Gln306	0.6	Val394	186.9
Gln308	0.6	Leu401	23.5
Gln310	0.6	Val405	9.4
Gln321	0.7	Val408	9.4
Val322	0.2	Gln410	1.2
Leu327	189.1	Val414	0.6
Phe330	203.7	Leu417	10.0
Ile333	1.4	Val418	0.6
Gln335	0.5	Ile425	0.1

Asterisks indicate two clusters of residues that could not be distinguished on gels; the half-lives of these residues thus default to the single, longest value (see text).

their relative reactivities to iodoacetamide in the native TIP47 protein (**Fig. 1b**, lanes 2–5).

We also monitored the reactivities of cysteine residues incorporated in place of valine, leucine or isoleucine (**Fig. 1c**). The half-life of the nonalkylated residues varied from <1 min (Ile386) to >600 min (Val366), corresponding to the degree of side chain burial in the native structure. We determined the half-lives of 48 cysteine probes in TIP47 (**Table 1**).

A protection factor can be estimated by comparing the rate constant for alkylation of a cysteine at a specific position to the alkylation rate constant for a fully exposed thiol[1]. Thus a protection factor represents the extent to which a residue is protected from alkylation. We calculated protection factors for 48 amino acid positions in TIP47 (**Fig. 1d**). A fully exposed thiol yielded a protection factor of 1, and rates up to 10^4-fold slower than the intrinsic rate were observed. Previous studies of triosephosphate isomerase showed that side chains with protection factors >20 were $<30\%$ solvent accessible in the crystal structure[1]. A high proportion of leucine (82%), isoleucine (50%) and valine (58%) positions showed protection factors above this limit (**Table 2**). This is consistent with the burial of hydrophobic residues in the TIP47 structure. As expected, a much lower fraction of glutamine sites (20%) showed protection factors >20. This is consistent with the enriched distribution of glutamines on the surface of native TIP47.

Based on 22 structures in the protein structure database[10], the expected probability for burial of specific amino acids in TIP47 was estimated to be 60–70% for valine, leucine and isoleucine residues and 17% for glutamine, in accordance with our data (**Table 2**). A much smaller fraction of phenylalanines were buried (25%) than was expected (59%). This may be explained by the low absolute number of phenylalanine residues in the protein, and/or by their possible enrichment at binding interfaces.

Table 2 | Relative burial of different amino acids in TIP47

	Leu	Ile	Val	Gln	Phe
Residues (total)	11	6	12	15	4
Residues with protection factor >20	9 (82%)	3 (50%)	7 (58%)	3 (20%)	1 (25%)
Predicted fraction buried (%)	62	67	66	17	59

Cysteine replaced leucine, isoleucine, valine, glutamine or phenylalanine at different positions in TIP47. Cysteine probes misincorporated at specific amino acids were scored for a protection factor >20 and compared with the expected representation of that residue to buried residues in native TIP47.

The probability of each residue occurring on the protein surface (**Fig. 1d**, lower panel) was aligned with the experimentally determined TIP47 protection factor profile (**Fig. 1d**, upper panel). Regions likely to be buried were predicted at residues 270–280, 325–335, 345–355 and 400–420, correlating well with high measured protection factors in native TIP47. Similarly, regions 285–295 and 305–325 were both predicted and shown experimentally to be solvent exposed. In contrast, TIP47 residues 355–400 had alternating high and low protection factors, correlating poorly with theoretical surface probability. An alternative prediction of buried and exposed residues using the PHD server[11] yielded similar results (data not shown). This observation demonstrates the power of MPAX to provide accurate surface topology.

Prediction of protein-protein interaction interfaces

We compared directly the protection factors of TIP47 residues with the contribution of specific amino acids to buried residues in 22 proteins of known structure[10] (**Fig. 2a**). As expected, amino acids with increased burial probability clustered at relatively higher protection factors. All TIP47 glutamine residue positions

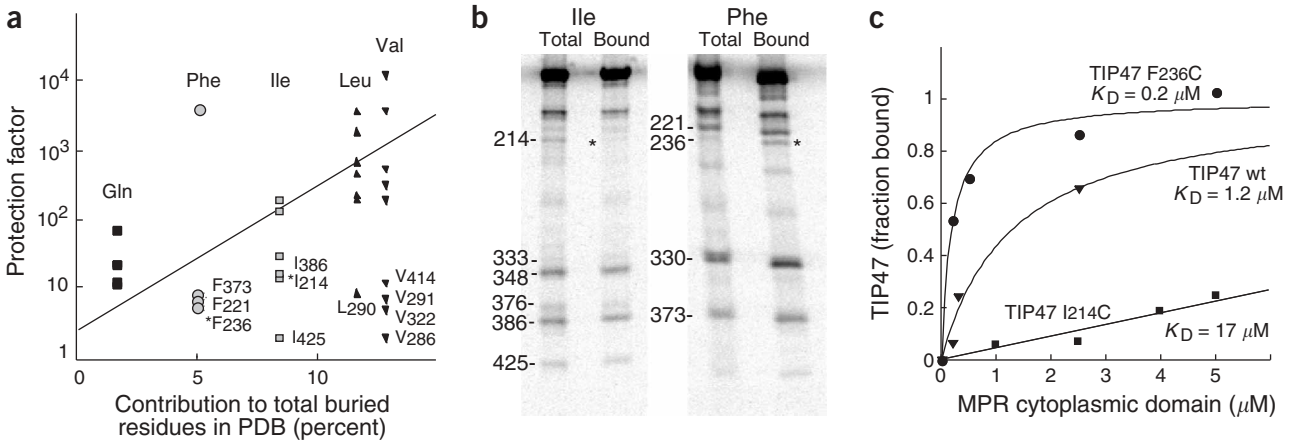

Figure 2 | Identification of protein interfaces. (**a**) Relationship between protection factor and amino acid burial probability. Protection factors are plotted against the relative contribution of specific amino acids at buried positions in 22 protein structures from the protein structure database (PDB)[10]. Burial probabilities are molar fractions (%) of amino acids in samples of 2,001 residues with <20 Å^2 accessible surface area. The data are shown for the glutamine (G), phenylalanine (F), isoleucine (I), leucine (L) and valine (V) positions. The line represents the expected correlation between protection factor and the burial propensity of residues in the protein data bank and is an exponential fit to protection factor averages of specific amino acids. Biochemically characterized TIP47 mutants (F236C and I214C) are indicated by asterisks. (**b**) Comparison of total versus MPR cytoplasmic domain affinity column–bound isoleucine and phenylalanine products. Specific residues are indicated numerically; asterisks indicate depletion of I214C and enrichment of F236C, respectively. Amino acid positions are given at left. (**c**) TIP47 single cysteine mutants show enhanced or decreased binding to the CI-MPR cytoplasmic domain. A constant, limiting amount of TIP47 was incubated with increasing amounts of CI-MPR cytoplasmic domain. Solid lines represent a fit to the equation for bimolecular reversible binding. The K_D was determined from the binding isotherm. Binding curves and constants are shown for wild-type TIP47, TIP47 containing a single Phe→Cys substitution at position 236 and TIP47 containing a single Ile→Cys substitution at position 214.

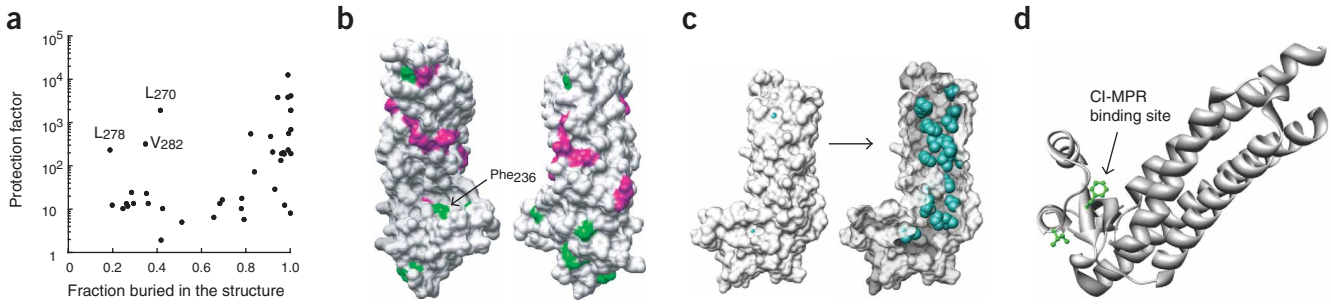

Figure 3 | Comparison of MPAX results with the TIP47 crystal structure. (**a**) Protection factors for each cysteine-misincorporation site are plotted against the fractional burial[15] of the corresponding wild-type residue in the TIP47 structure. (**b**) MPAX-predicted surface residues with protection factors <100 are shown in relation to the TIP47 structure (magenta and green). Green residues are surface-localized, hydrophobic residues predicted to participate in protein-protein interactions (from lower right quadrant of **Fig. 2a**). Phe236, which when mutated to cysteine shows enhanced MPR affinity, is indicated. (**c**) MPAX-predicted buried residues with protection factors >100 (turquoise) are shown in relation to a sliced-open representation (right) of the TIP47 structure (left). (**d**) Residues influencing the affinity of TIP47 for CI-MPR cytoplasmic domains (Phe236 and Ile214) are shown in green.

clustered in the left bottom quadrant of this plot. Phenylalanine residues clustered at protection factors <10, whereas isoleucine, leucine and valine residues clustered at higher protection factors (10–200, 200–13,000 and 200–10^4, respectively).

Readily identified in this graph were a group of TIP47 residues with high theoretical burial probability but low protection factors (**Fig. 2a**, lower right), including Ile214, Phe221, Phe236, Val286, Leu290, Val291, Val322, Phe373, Ile386, Val414 and Ile425. These are excellent candidates for residues that contribute hydrophobic interactions with TIP47 binding partners. As discussed later, mutations of Ile214 and Phe236 altered TIP47 affinity for MPR cytoplasmic domains.

Selection for TIP47 mutants with altered binding to MPRs

We screened TIP47 mutant pools containing randomly misincorporated cysteines for an alteration in their ability to bind to the cytoplasmic domain of the cation-independent MPR (CI-MPR) bound to a resin support. Proteins with increased interaction affinity should be enriched in the column-bound material; a single TIP47 mutant (F236C) was 3-fold more abundant in the receptor-bound fraction. Proteins with decreased affinity will be underrepresented. TIP47 I214C was 5-fold less abundant, as compared to total TIP47 (**Fig. 2b**). These mutants were shown to have a 6-fold greater (K_D ~0.2 μM) versus a 14-fold lower (K_D ~17 μM) affinity for the CI-MPR cytoplasmic domain as compared to wild-type TIP47 (**Fig. 2c**). In summary, a search for surface-localized, hydrophobic residues is likely to represent a powerful means to identify residues in protein interfaces. In our experiments, a single affinity chromatography step was sufficient to confirm them.

Validation with TIP47 structure

After submission of this manuscript, the three-dimensional structure of a large portion of TIP47 (ref. 7) became available to us. Satisfyingly, our data agree very well with the structure. MPAX protection factors correlated well with physical burial of amino acid side chains (**Fig. 3a**). Completely buried side chains had the highest protection factors; partly buried side chains had significantly lower protection factors. The correlation was strongest for fully buried residues, highlighting the fact that MPAX detects dynamics of a breathing molecule in solution, whereas crystallography detects an immobile structural form.

Residues predicted to be surface localized are indeed present on the TIP47 surface (**Fig. 3b**); these include 8 of the 11 hydrophobic residues (green) predicted to be important for partner protein binding (**Fig. 2a**). The other three such residues were not discernible in the structure[7], most probably because of their presence in a flexible connecting loop. Residues predicted to be buried can, for the most part, only be seen in the structure when it is sliced open (**Fig. 3c**).

Only 3 of the 48 residues examined did not show a general correlation of burial with protection factor (Leu270, Leu278, Val282; **Fig. 3a**). Specifically, misincorporated cysteines at Leu269, Leu270 and Leu272 and cysteines at Leu276 and Leu278, were not distinguishable by our method owing to the limited resolving power of tricine gels. In these cases, all residues default to the highest protection factor of the cluster (**Table 1**). Thus, where residues of the same type are close together in sequence, the protection factor of an exposed residue may be overestimated. Despite this limitation, the correlation was excellent overall. Finally, our method indicated that Val282 is protected, whereas it was surface localized in the structure[7]. The reported structure, however, did not include residues 285–316. We believe that these additional residues are likely to protect Val282 in solution. The Val282 side chain is oriented toward the core of the protein[7] and could easily be covered by the residues not discerned by crystallography.

The positions of residues selected by affinity chromatography for enhanced (Phe236) or reduced (Ile214) MPR binding are shown (**Figs. 3b,d**). Phe236 is located prominently on the floor of a deep hydrophobic cleft (**Fig. 3b**) that was noted to be the most striking feature of the TIP47 structure and suggested as a site for protein-protein interactions[7]. In contrast, Ile214 is located on the opposite side of the cleft; it is directly connected to a loop in the structure that contributes a wall to the cleft (**Fig. 3d**). We propose that changing this residue to cysteine either alters the disposition of the loop or changes the shape of the cleft, thereby decreasing MPR binding affinity.

DISCUSSION

We have used MPAX[1] to screen an entire polypeptide sequence for surface-localized amino acid residues and have shown here that this method can accurately predict protein-protein interaction interfaces. Combined with affinity chromatography, MPAX permitted

rapid *in vitro* selection of mutant proteins with alterations in protein-protein interaction. Our approach led to the identification of two mutant TIP47 proteins that show enhanced (F236C) and decreased (I214C) binding, respectively, to MPR cytoplasmic domains. Both of these residues are surface localized and thus appropriate candidates for an MPR cytoplasmic domain–binding interface. Moreover, comparison of our data with the recent protein structure[7] validated our approach.

Aromatic and hydrophobic residues are enriched at binding interfaces[12]; moreover, phenylalanine and isoleucine are twice as common in interface areas than in the total solvent-accessible surface area of protein-protein complexes, consistent with our findings. Compilations of the amino acid compositions of protein cores of known protein structures provide a relative propensity of an amino acid to be found buried in folded proteins[10]. By comparing this propensity with experimentally obtained protection factors for amino acid residues of a protein of interest, one can quickly identify candidate residues that are more solvent exposed than would be expected. Such residues are likely to contribute to protein-protein binding interfaces. In the present study, Phe236 is the only solvent-exposed, evolutionarily conserved phenylalanine. Consideration of residue conservation can thus further enhance the predictive power of this approach.

We have mapped the relative surface disposition of cysteine residues inserted into the protein at positions normally occupied by leucine, isoleucine, valine, glutamine or phenylalanine. We believe that the misincorporated cysteine residues reflect the normal topology of the replaced residues for several reasons. First, cysteine is small and neutral and can easily be accommodated either within the hydrophobic core of a folded protein or on the surface. Second, the specific mutants we generated show normal overall structure as determined by circular dichroism, and their abilities to interact with specific binding partners are retained, if not enhanced. Third, the relative burial of cysteine residues in the folded TIP47 is highly consistent with the expected burial of the particular amino acids that we have replaced (**Table 2**). In only 1 of 48 positions did a structurally, fully buried residue seem to be surface localized by MPAX. In this case, mutation of Phe373 to cysteine appeared to locally unfold the protein by removing a residue side chain that is key to the stability of a four-helix bundle. Nevertheless, the mutant protein still bound to the MPR (**Fig. 2b**).

Unfortunately, our mutant residue set was not protected from alkylation by protein-protein interaction. MPAX has been used to identify protein-protein interaction interfaces[1]; we hope that the use of additional misincorporator tRNAs will make this possible for these protein pairs in the future.

In summary, we have presented a powerful means to identify surface residues in proteins whose three-dimensional structure has yet to be determined. Identification of solvent-exposed hydrophobic residues can reveal key sites for protein-protein interaction. Combining MPAX with affinity chromatography provides a rapid method for *in vitro* selection of affinity-altered mutant proteins.

METHODS

Gene assembly. A synthetic gene corresponding to residues 152–434 of human TIP47 was assembled[13] using a single codon corresponding to the cysteine-misincorporator tRNA anticodons used for each amino acid. The single, naturally occurring cysteine codon at TIP47 position 341 was changed to an alanine codon. The assembled gene was cloned into *pET28a pH6_PKA* to provide an N-terminal histidine tag and a C-terminal protein kinase A recognition site, generating *pH6_TIP47_PKA*[1].

Cysteine misincorporation. BL21(DE3) cells were cotransformed with *pH6_TIP47_PKA* and different plasmids carrying mutant *E. coli* cysteine tRNAs with anticodon triplets corresponding to either valine, leucine, isoleucine, phenylalanine, tryptophan or glutamine[1]. Cells expressing TIP47 were grown in M63 media, induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside at an OD_{600} of 0.2–0.3, and purified[4]. Misincorporation frequencies were as reported previously[1].

Radiolabeling of TIP47. TIP47 (1–130 μg) was radiolabeled for 15 min at 37 °C in a reaction containing 1 mM unlabeled ATP, 132 nM aqueous [γ-^{33}P]ATP (3,000 Ci/mmol) (ICN), 3 U protein kinase A (Sigma) and 6 mM dithioerythritol in a 100-μl volume. For K_D determinations, proteins were bound to a Ni-NTA resin (Qiagen) and supplemented with 10 μg/ml BSA. Nucleotides and protein kinase A were removed by washing in buffer A (50 mM HEPES, pH 7.5, 150 mM KCl, 1 mM $MgCl_2$, 10% (w/v) glycerol and 10 μg/ml BSA) on a supported syringe and eluted in 250 mM imidazole. Eluted TIP47 was counted in an LS 5000 CE Liquid Scintillation System (Beckman) and the specific activity was determined after protein assay.

Chemical cleavage by NTCB (2-nitro-5-thiocyanobenzoic acid). Chemical cleavage was as described[1] with some modification. Purified TIP47 carrying random misincorporated cysteines was radiolabeled. An equal volume of 8 M guanidine HCl, 200 mM sodium bicine (pH 8.6), 50 mM NTCB was added for 5 min at 22 °C. Proteins were precipitated by addition of 1 ml 0.05% sodium deoxycholic acid and 100 μl 50% trichloroacetic acid. Pellets were washed twice in 600 μl acetone and dried in a speed vacuum centrifuge. Proteins were cleaved, resolved and quantified[1].

Alkylation rate determination. Radiolabeled TIP47 (1 μg) was combined with 100 mM bicine (pH 8.6), 10 mM iodoacetamide and incubated at 22 °C in a 450-μl volume. Aliquots (100 μl) were removed and quenched after 1, 5, 20 or 50 min with 100 μl, 25 mM β-mercaptoethanol and 0.1 mg/ml BSA. TIP47 was also incubated and quenched in the absence of iodoacetamide or in the presence of 2.4 M guanidine HCl to generate nonalkylated or fully alkylated thiols, respectively. Proteins were denatured and chemically cleaved. Amounts of protein corresponding to equal counts were loaded onto tricine gels and bands were quantified. Results represent at least four independent experiments.

Intrinsic alkylation rate determination. TIP47 carrying cysteine substitutions at isoleucine positions was alkylated with 0.8 mM iodoacetamide in 2.6 M guanidine HCl as described above. Reactions without iodoacetamide were quenched immediately and those with iodoacetamide were quenched after 30 or 60 s. To achieve complete thiol alkylation, the iodoacetamide concentration was increased to 10 mM. The second-order rate constant was 35 $M^{-1}s^{-1}$, as determined by averaging the decay rates of five cysteine thiol probes in the unfolded protein.

***In vitro* selection of TIP47 I214C and F236C.** Radiolabeled TIP47 cysteine mutant pools (3.5 μM) were bound to GST-CI-MPR

cytoplasmic domain (11 μM) in 200 μl total volume with 1 mg/ml BSA for 50 min at 22 °C. Complexes were bound to 50 μl (1:1 slurry) glutathione–Sepharose 4B (Amersham) for 30 min at 22 °C, washed, and eluted in 200 μl 8 M guanidine HCl, 100 mM sodium bicine (pH 8.6). Proteins were cyanylated and cleaved as described above. Samples were counted and equal amounts of radioactivity loaded on a tricine gel. Total receptor-bound TIP47 was also cyanylated, cleaved and loaded. Band intensity corresponding to different residue positions was quantified and compared internally within a single sample. Relative band intensities were then compared for total and receptor-bound samples.

TIP47 binding to GST-CI-MPR. Radioactively labeled TIP47 (60 nM for F236C or 0.5 μM for I214C) was bound to purified GST-CI-MPR cytoplasmic domain (residues 13–163)[4] as described[5]. Eluted TIP47 was counted and the receptor bound fraction calculated.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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