

Protein photo-cross-linking in mammalian cells by site-specific incorporation of a photoreactive amino acid

Nobumasa Hino^{1,2}, Yuko Okazaki^{1,2,4}, Takatsugu Kobayashi¹, Akiko Hayashi², Kensaku Sakamoto^{1,2} & Shigeyuki Yokoyama¹⁻³

We report a method of photo-cross-linking proteins in mammalian cells, which is based on site-specific incorporation of a photoreactive amino acid, *p*-benzoyl-L-phenylalanine (*p*Bpa), through the use of an expanded genetic code. To analyze the cell signaling interactions involving the adaptor protein Grb2, *p*Bpa was incorporated in its Src homology 2 (SH2) domain. The human *GRB2* gene with an amber codon was introduced into Chinese hamster ovary (CHO) cells, together with the genes for the *Bacillus stearothermophilus* suppressor tRNA^{Tyr} and a *p*Bpa-specific variant of *Escherichia coli* tyrosyl-tRNA synthetase (TyrRS). The Grb2 variant with *p*Bpa in the amber position was synthesized when *p*Bpa was included in the growth medium. Upon exposure of cells to 365-nm light, protein variants containing *p*Bpa in the positions proximal to the ligand-binding pocket were cross-linked with the transiently expressed epidermal growth factor (EGF) receptor in the presence of an EGF stimulus. Cross-linked complexes with endogenous proteins were also detected. *In vivo* photo-cross-linking with *p*Bpa incorporated in proteins will be useful for studying protein-protein interactions in mammalian cells.

To explain the molecular mechanisms underlying cell activities, it is important to analyze the network of interactions between biomolecules. The yeast two-hybrid system^{1,2} and protein arrays^{3,4} have been developed to find possible interactions between a protein of interest and other proteins. Because these techniques are based on the behavior of proteins *in vitro* or in non-native cellular contexts, the interactions identified using these techniques have been examined to determine whether they occur in native cells. Methods involving coimmunoprecipitation and precipitation with tagged proteins can isolate the protein complexes that actually form in cells, and are thus used extensively for analyses of *in vivo* interactions. For these methods to be effective, however, the complexes must be stable and cannot dissociate during the cell extract preparation.

Cross-linkers create covalent bonds between the bound biomolecules, and thus even the complexes formed by weak interactions can be isolated. Therefore, cross-linking reagents are useful even for analyses of protein–nucleic acid interactions *in vitro*. Cross-linkers can be incorporated into peptides and proteins; amino acids with photoreactive linkers in their side-chain moieties^{5,6}, such as *p*Bpa (Fig. 1a), can be incorporated into defined positions in synthetic peptides^{7,8} or proteins synthesized *in vitro*^{9,10}. Upon excitation at 350–365 nm, the benzophenone group of *p*Bpa reacts with nearby C–H bonds. The feasibility of *in vivo* photo-cross-linker incorporation into proteins using *E. coli* cells with an expanded genetic code has been demonstrated^{11,12}. A variant of *Methanococcus jannaschii* TyrRS that specifically recognizes *p*Bpa had been expressed in *E. coli*, together with a suppressor tRNA derived from *M. jannaschii* tRNA^{Tyr}. This TyrRS variant aminoacylates only *p*Bpa, and this tRNA is not recognized by any endogenous aminoacyl-tRNA synthetase (aaRS; this principle is known as ‘orthogonality’). Thus, *p*Bpa had been assigned to the amber codon in the *E. coli* cells. The addition of *p*Bpa to the growth medium had resulted in synthesis of glutathione *S*-transferase (GST) with *p*Bpa in cells in which one codon in the gene encoding GST was replaced with an amber codon. The cross-linked dimer, in which the two GST molecules had been covalently bound to each other, was isolated from the cells after an exposure to 365-nm light for up to 30 min.

The application of this method to mammalian cells requires an orthogonal tRNA-aaRS pair for *p*Bpa: a *p*Bpa-specific TyrRS variant that does not recognize any mammalian tRNAs and a suppressor tRNA that is only recognizable by this variant aaRS. The *p*Bpa-specific variant of *M. jannaschii* TyrRS is inappropriate for use in a mammalian system because it can aminoacylate the mammalian tRNA^{Tyr} species¹³. The orthogonality of prokaryotic tRNA-aaRS pairs in eukaryotic translation has been demonstrated with the *E. coli* pair of amber suppressor tRNA^{Gln} and glutaminyl-tRNA synthetase¹⁴ and subsequently with the tRNA^{Tyr}-TyrRS pair¹⁵. Based on the latter observation, we had developed an

¹Department of Biophysics and Biochemistry, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. ²RIKEN Genomic Sciences Center, 1-7-22 Suehiro-cho, Tsurumi, Yokohama 230-0045, Japan. ³RIKEN Harima Institute at SPring-8, 1-1-1 Kohto, Mikazuki-cho, Sayo, Hyogo 679-5148, Japan. ⁴Present address: Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan. Correspondence should be addressed to S.Y. (yokoyama@biochem.s.u-tokyo.ac.jp).

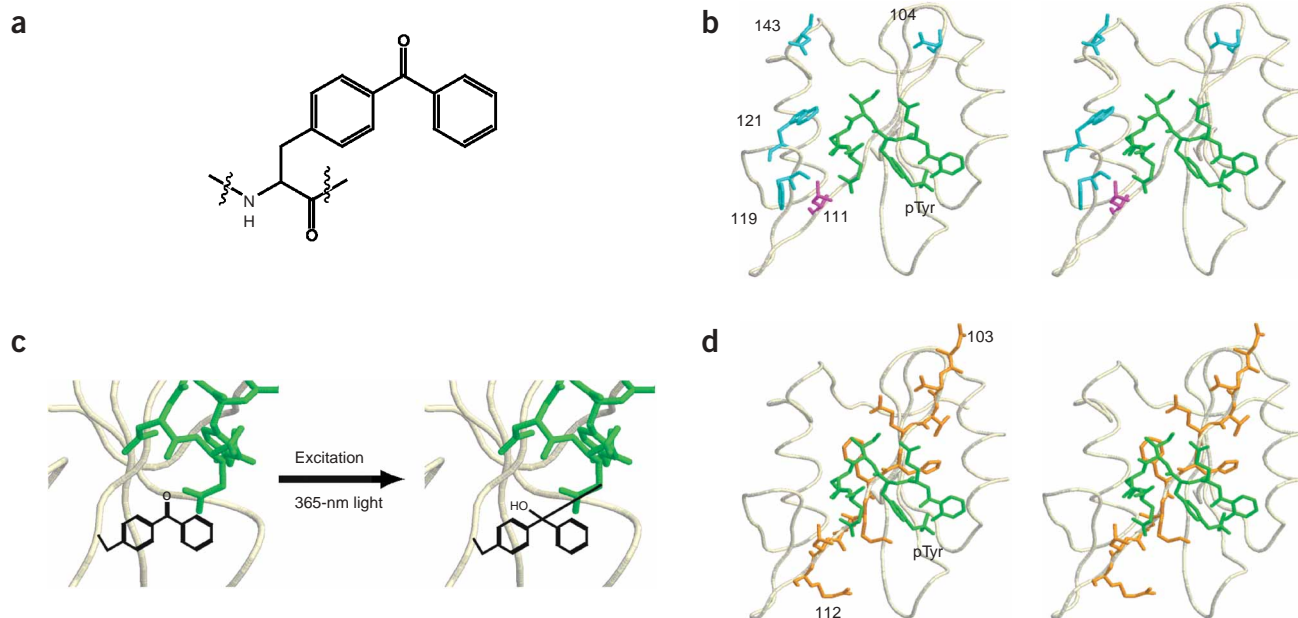


Figure 1 | Site-specific incorporation of *pBpa* in the Grb2 SH2 domain. (a) Chemical structure of a *pBpa* residue. The crystal structure (stereo view) of the complex between the Grb2 SH2 domain and a peptide inhibitor with the sequence Glu–phospho-Tyr–Ile–Asn–Gln (in green), which corresponds to residues 1067–1071 of the EGF receptor²⁰. (b) pTyr, phosphotyrosine. Residue 111 is shown in magenta and residues 104, 119, 121 and 143 in cyan. (c) Illustration of a photo-cross-link between *pBpa* in position 111 of Grb2 and a bound protein. (d) The crystal structure (stereo view) of the complex between the Grb2 SH2 and the peptide inhibitor, in which residues 103–112 on the β D and β D' strands²⁰ are shown in orange.

amber suppression system for the site-specific incorporation of a non-natural amino acid into proteins in mammalian cells¹⁶. This system comprises an *E. coli* TyrRS variant specific to 3-iodo-L-tyrosine and a suppressor tRNA derived from *B. stearotherophilus* tRNA^{Tyr}, and specifically incorporates this amino acid into amber positions, with an occupancy of >95%, in CHO cells. Recently, a *pBpa*-specific variant of *E. coli* TyrRS (*Eco-pBpaRS*) has been developed¹⁷. We thought that this variant could be used, in combination with our system, for the incorporation of *pBpa* into proteins in mammalian cells.

In the present study, we showed that the *Eco-pBpaRS* and the *B. stearotherophilus* suppressor tRNA^{Tyr} can be used as an orthogonal pair for site-specific incorporation of *pBpa* into proteins in CHO cells. *pBpa* was incorporated into the ligand-binding pocket of the SH2 domain of the adaptor protein Grb2. The *pBpa*-containing Grb2 proteins were cross-linked by 365-nm light to the transiently expressed EGF receptor in the presence of EGF stimuli, as well as to endogenous proteins such as ErbB2. Therefore, the *in vivo* photo-cross-linking obtained using the expanded genetic code is useful for studying protein-protein interactions in mammalian cells.

RESULTS

Site-specific incorporation of *pBpa* in Grb2 SH2

Grb2 is an adaptor protein that mediates the extracellular signals to the Ras protein by binding to various target molecules, including the members of the EGF receptor family^{18,19}. This Grb2 accommodation by the EGF receptor involves the SH2 domain of Grb2, and this domain directly binds Tyr1068 of the receptor, which becomes phosphorylated upon EGF stimulation. The tertiary structures of this Grb2 SH2 domain, complexed with a ligand

peptide, have been determined by X-ray crystallography and NMR^{20–22}. The candidates for the amino acid to be replaced by *pBpa* are the residues that are located in the neighborhood of the bound ligand but are not involved in the binding. Several candidates were selected based on the crystal structure (Fig. 1), and we first changed position 111.

The *GRB2* gene variant containing an amber codon in this position, *GRB2*(Am111), was tagged with a Flag peptide and introduced into CHO cells, together with the genes for *Eco-pBpaRS* and the *B. stearotherophilus* suppressor tRNA^{Tyr}. The Grb2 variant with *pBpa* was produced depending on both the expression of the suppressor tRNA–*Eco-pBpaRS* pair and the presence of *pBpa* in the growth medium, but at a lower level than the wild-type Grb2 (Supplementary Fig. 1 online). Thus, *pBpa* was incorporated into the amber position of *GRB2*(Am111).

Binding of the *pBpa*-containing Grb2 to the EGF receptor

We used coimmunoprecipitation experiments to determine whether the Flag-tagged Grb2(*pBpa*111) could still bind to the EGF receptor in spite of the presence of the bulky *pBpa* residue near the ligand binding site. This receptor is not endogenously expressed in CHO cells²³ and was therefore introduced transiently. Using a specific antibody against this receptor, we detected products of two different molecular masses, 150 and 170 kDa (Fig. 2a; lanes 1–4); the product with the lower mass had previously been reported to be a nonphysiological byproduct that resulted from overproduction of the receptor in CHO cells²⁴. EGF treatment facilitated the coprecipitation between the 170-kDa EGF receptor and the wild-type Grb2, and this precipitated receptor was phosphorylated at Tyr1068 (Fig. 2b,c; lanes 1,2). By contrast, the 150-kDa EGF receptor byproduct was phosphorylated at Tyr1068 and

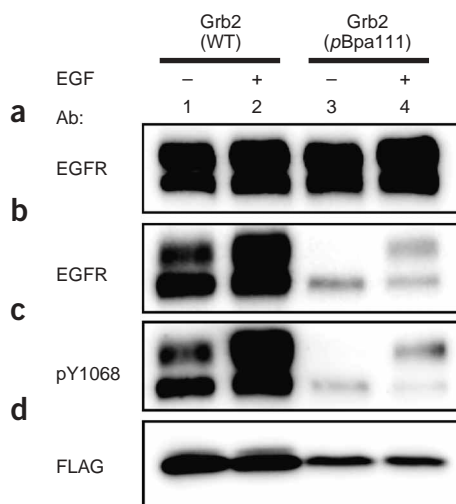


Figure 2 | Analysis of the interactions between Grb2 and the EGF receptor by coimmunoprecipitation. The wild-type Grb2 or Grb2(*pBpa111*) was expressed together with the EGF receptor in CHO cells, either treated with EGF or not treated. Ab, antibody. (a) A 20- μ g aliquot of the cell lysate was analyzed with the antibody against the EGF receptor. (b–d) An immunoprecipitate with the anti-Flag antibody from 1.0 mg of the cell lysate was probed with antibodies against the EGF receptor (b), phospho-Tyr1068 (c) and the Flag tag (d).

immunoprecipitated with the anti-Flag antibody, but independently of EGF treatment (Fig. 2b,c; lanes 1,2).

When Grb2(*pBpa111*) was expressed in place of the wild-type Grb2, the EGF receptor still immunoprecipitated with this Grb2 variant (Fig. 2b,c; lanes 3,4). Less of the immunoprecipitated receptor was obtained with the Grb2 variant than with the wild-type Grb2. This was probably because the cellular abundance of Grb2(*pBpa111*) was lower than that of the wild-type Grb2 (Fig. 2d). Thus, we concluded that Grb2(*pBpa111*) retains the ability to bind phospho-Tyr1068 on the EGF receptor.

Cross-linking between Grb2(*pBpa111*) and the EGF receptor

For photo-cross-linking between the EGF receptor and Grb2(*pBpa111*) complex, formed as a result of autophosphorylation on the tyrosine residues, the CHO cells expressing these molecules were stimulated with EGF and then immediately washed with ice-cold phosphate-buffered saline (PBS) before exposure to 365-nm light for up to 30 min on ice. Note that the internalization of the EGF receptor does not occur at 4 °C (ref. 25). Extracts of the cells were then prepared and treated with protein tyrosine phosphatase 1B, to dissociate the EGF receptor–Grb2(*pBpa111*) complexes that were not cross-linked. Thus, in addition to the cross-linked complex, Grb2(*pBpa111*) alone was expected to be precipitated with the anti-Flag antibody.

We detected a precipitate with a molecular mass of nearly 200 kDa after 15–30 min of light exposure when Grb2(*pBpa111*) was expressed (Fig. 3a; lanes 8,9) but did not detect such a precipitate when the wild-type Grb2 was expressed (Fig. 3a; lanes 1–5). We concluded that this product was a cross-linked complex between the Grb2 variant and the EGF receptor for the following reasons. First, the detection of this product was dependent on the EGF stimulus. Second, its molecular mass corresponds to the sum of those of the EGF receptor (170 kDa) and Grb2 (25 kDa). Third, it

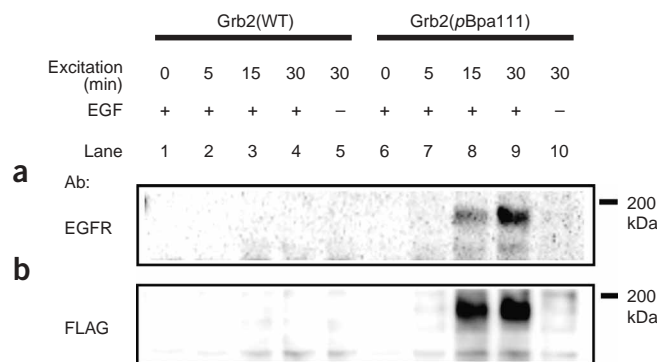


Figure 3 | Photo-cross-linking of Grb2(*pBpa111*) with the EGF receptor. (a,b) The wild-type Grb2 or Grb2(*pBpa111*) was expressed together with the EGF receptor in CHO cells, treated or not treated with EGF, and excited with 365-nm light. The light exposure was maintained for 0, 5, 15 and 30 min. An immunoprecipitate with the anti-Flag antibody from the cell lysate (0.2 mg) was probed with the anti-EGF receptor antibody (a) and the anti-Flag antibody (b).

was detected by both the anti-EGF receptor antibody and the anti-Flag antibody (Fig. 3b; lanes 8–9). Fourth, its detection depended on both the light exposure and the presence of *pBpa* in Grb2.

Grb2 variants containing *pBpa* at different positions

We then examined if any positions other than 111 could also be used for the cross-linking, by incorporating *pBpa* at different positions close to where the ligand bound in the crystal structure (Fig. 1b,d). After 15 min of light exposure, the cross-linked complexes between the EGF receptor and Grb2(*pBpa121*) or Grb2(*pBpa143*) (Fig. 1b) were detected by the anti-EGF receptor antibody and an anti-Grb2 antibody. The signals for these complexes were weaker than that between the EGF receptor and Grb2(*pBpa111*) (Fig. 4a,b; lanes 11, 14 and 5, respectively). When *pBpa* was incorporated in positions 104 and 119, which are more distant from the bound ligand than positions 111, 121 and 143, a weak signal for the cross-linked complex was detected for Grb2(*pBpa104*), but no signal was obtained for Grb2(*pBpa119*). The expression levels of these five Grb2 variants were similar to each other (Fig. 4c). This indicated that the efficiency of cross-linking correlates with the distance between the *pBpa* position and the bound ligand, and several positions in SH2 are useful for cross-linking.

Next, *pBpa* was systematically incorporated at positions 103 to 112 on the β D and β D' strands extending across the bound ligand²⁰ (Fig. 1d). Positions 107–111 are in the vicinity of the ligand, and after exposure to light, Grb2(*pBpa109*) as well as Grb2(*pBpa111*) gave a strong signal corresponding to the cross-linked complex, as detected by the anti-Grb2 antibody (Fig. 4d,e). The signals of cross-linked products were also detected by the anti-EGF receptor antibody (data not shown). On the other hand, a weak signal was obtained for Grb2(*pBpa108*), and no signal was detected for Grb2(*pBpa107*) and Grb2(*pBpa110*) (Fig. 4d). Grb2 His107 directly interacts with the phosphotyrosine residue in the ligand peptide, and its replacement by *pBpa* impaired the interaction between Grb2 and the receptor, as indicated by coimmunoprecipitation assay (Fig. 4f). Grb2 Phe108 and Val110 side chains are oriented inward and away from the ligand peptide (Fig. 1d), and in

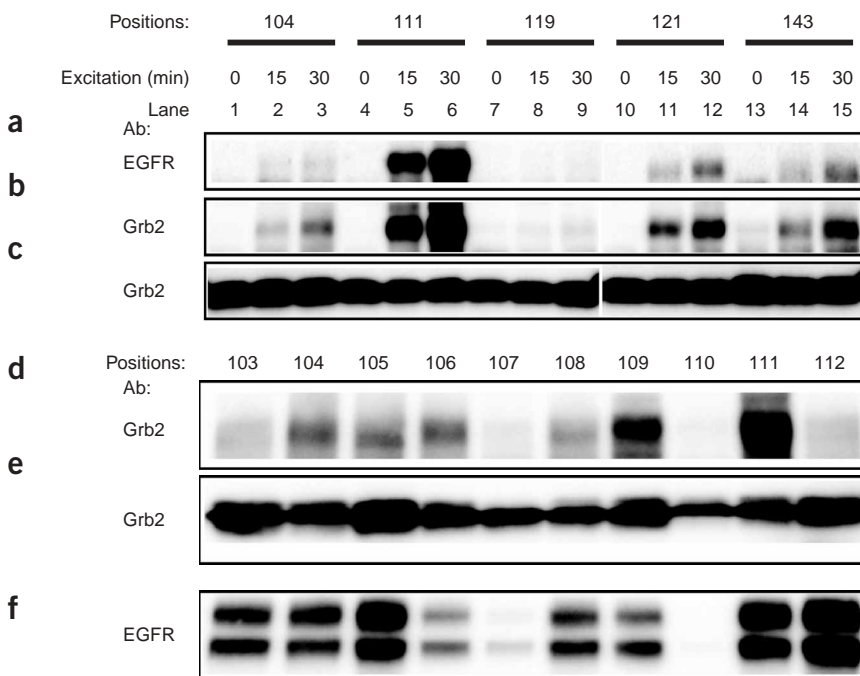


Figure 4 | Photo-cross-linking of Grb2 variants, containing pBpa at different positions in the SH2 domain, with the EGF receptor. (a–f) The Grb2 variants with pBpa at the indicated positions were expressed together with the EGF receptor in CHO cells, which were exposed to the 365-nm light for the indicated amounts of time. The cross-linked products (200 kDa), immunoprecipitated with the anti-Flag antibody from the cell lysate (0.5 mg for a–c, 1 mg for d–e), were probed with the anti-EGF receptor antibody (a) and the anti-Grb2 antibody (b,d). The uncrosslinked Grb2 (25 kDa) variants in these immunoprecipitates were also detected with the anti-Grb2 antibody (c,e). The abilities of the Grb2 variants to bind the EGF receptor were analyzed by means of coimmunoprecipitation between the variants and the receptor; the immunoprecipitate with the anti-Flag antibody from the lysate of the unexposed cells (0.5 mg) was probed with the anti-EGF receptor antibody (f).

addition, the pBpa incorporation at position 110 reduced the expression level of the Grb2 variant (Fig. 4e). Weak signals were obtained for Grb2(pBpa104), Grb2(pBpa105) and Grb2(pBpa106), which have alterations in residues that are at an intermediate distance from the bound ligand. As for the distal positions (103 and 112), neither Grb2(pBpa103) nor Grb2(pBpa112) formed a cross-linked complex with the receptor. These results demonstrated the applicability of the present method in analyzing the interface between interacting proteins.

Cross-linking of Grb2(pBpa111) with endogenous proteins

Finally, we examined whether the Grb2-Flag containing pBpa at position 111 cross-linked with endogenous proteins. Unstimulated, resting CHO cells expressing Grb2(pBpa111) were exposed to 365-nm light and the cell extracts were precipitated with the anti-Flag antibody. After separation by SDS-PAGE, the staining of these precipitates with the anti-Grb2 antibody should allow us to detect Grb2 and its cross-linked complexes. We used this antibody, which is different from the antibody used for the precipitation, to identify cross-linked products. Bands corresponding to complexes between Grb2(pBpa111) and certain endogenous proteins were observed, depending on the exposure to light, whereas no such light-dependent bands were detected when the Flag-tagged wild-type Grb2 was expressed (Fig. 5a). Most of the bands corresponding to the cross-linked complexes approached the maximal levels within 15 min. In particular, we focused on a faint band at about 200 kDa, because the molecular mass corresponds to that of the complex with another member of the EGF-receptor family, ErbB2,

which is endogenously expressed in CHO cells²³. The band of the putative cross-linked complex between ErbB2 and Grb2(pBpa111) was detected by staining with an anti-ErbB2 antibody (Fig. 5b). Under resting conditions (serum starvation for 12 h), ErbB2 is only slightly tyrosine-phosphorylated (data not shown). Using the anti-ErbB2 antibody, we also detected the band corresponding to the un-cross-linked ErbB2 (185 kDa), which coimmunoprecipitated with Grb2-Flag, owing to the omission of the phosphatase

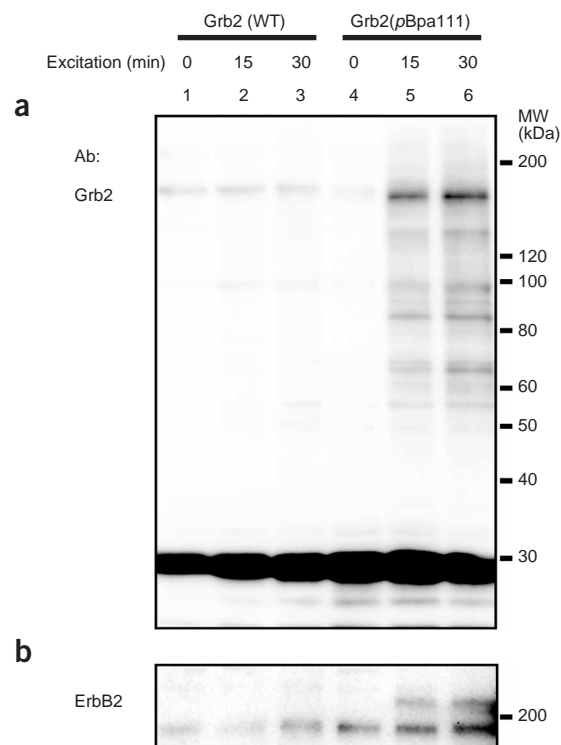


Figure 5 | Photo-cross-linking of Grb2(pBpa111) with endogenous proteins. (a,b) The wild-type Grb2 or Grb2(pBpa111) was expressed in CHO cells. A 365-nm light exposure was performed for the indicated amounts of time. Immunoprecipitates with the anti-Flag antibody from the cell lysates (1.6 mg) were probed with the anti-Grb2 antibody (a) and the anti-ErbB2 antibody (b).

treatment (Fig. 5b). The band intensities of the cross-linked and un-cross-linked ErbB2 molecules suggested that about 30% of the ErbB2-Grb2 complexes were cross-linked. Therefore, the Grb2 cross-linking efficiency seems to be higher for endogenous proteins than for the overexpressed EGF receptor.

DISCUSSION

Pairs of a suppressor tRNA and an aaRS variant with an engineered amino acid specificity have been expressed in bacterial, yeast and mammalian cells to expand the repertoire of amino acids incorporated in proteins^{16,17,26–28}. This technology had been used for photo-cross-linking of the pBpa-containing GST homodimer in *E. coli* cells^{11,12}. Our results demonstrate that cell signaling proteins, either overexpressed or endogenous in mammalian cells, can be successfully cross-linked and detected by using pBpa. Consequently, *in vivo* cross-linking, based on the genetic code expansion, can also be applied to study a variety of protein-protein interactions. The cross-linked protein complexes are stable because of the formation of a covalent bond, and thus their dissociation during cell extract preparation can be avoided. This advantage will facilitate the isolation of complexes formed by transient or weak interactions between proteins, even if some of these interactions are not detectable by traditional coimmunoprecipitation. Application of our method to the discovery of protein-protein interactions will benefit from the recent developments in mass spectrometry as well as in tandem affinity purification—in which two distinct affinity columns are used for isolating cross-linked products²⁹—because the cross-linked products could be analyzed by peptide mass fingerprinting to identify the target proteins.

In addition, because the covalent bonds are formed in the cells as a consequence of exposure to 365-nm light, the cellular interactions can be distinguished from the false interactions that could occur during cell extract preparation or incubation with antibodies. For example, when the EGF receptor is overproduced in CHO cells, a nonphysiological byproduct, the 150-kDa EGF receptor, is observed in addition to the physiological 170-kDa EGF receptor²⁴. In the present study, the 150-kDa EGF receptor was coimmunoprecipitated with Grb2 both with and without EGF treatment, but was not cross-linked at all to Grb2(pBpa111) (Figs. 2 and 3). Presumably, the byproduct was not properly expressed on the cell surface and was unable to bind Grb2, even with the EGF-independent Tyr1068 phosphorylation, and the observed coimmunoprecipitation with Grb2 was due to nonspecific binding that occurred during or after the cell extract preparation. Thus, the present method specifically detects the natural complexes formed in the cellular environment. The specificity of this protein-directed cross-linking contrasts starkly with the random cross-linking obtained with cell membrane-permeable cross-linkers³⁰.

Protein cross-linking has been used to determine the position of a protein relative to those of other proteins within a macromolecular complex³⁰; the formation of cross-links between two proteins indicates their direct contact within the complex, with no intervening factors. The site-specific incorporation of cross-linker amino acids can pinpoint the sites on a protein in the vicinity of the interacting proteins. The present expanded genetic code method does not require the elimination of all of the protein's cysteine residues aside from the one at the target site, as is the case with the existing method³¹. The extent of cross-linking depends on the distance between the pBpa residue and the target protein⁶. Our

results with pBpa in various positions of Grb2 correlate well with the distances observed in the crystal structure of Grb2 complexed with a ligand peptide.

In the absence of structural information about a protein, pBpa can be incorporated in proximity to the conserved amino acids or the amino acids known to be essential for ligand binding. In the present study, we tested the feasibility of this method; pBpa was systematically introduced at ten sequential positions around His107, which is highly conserved in SH2 domains²⁰, and five of the Grb2 variants were photo-cross-linked to the EGF receptor. Alternatively, pBpa could be incorporated into the putative binding domain at intervals of several residues.

METHODS

Materials. pBpa was purchased from Bachem AG. The anti-Flag M2 antibody was from Sigma, the anti-hemagglutinin antibody was from Roche Diagnostics, the anti-EGF receptor antibody (against its intracellular domain) and the anti-Grb2 antibody (against its C-terminal domain) were from Santa Cruz Biotechnology, and the antibody against the phospho-Tyr1068 in the EGF receptor was from Cell Signaling Technology. The horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG antibodies were both from Amersham Biosciences.

Site-directed mutagenesis and plasmid construction. The gene encoding *Eco*-pBpaRS was generated by site-directed mutagenesis of the wild-type TyrRS gene from *E. coli*, which was achieved by PCR with mutagenic primers to replace Tyr37, Asp182 and Leu186 by glycine, glycine and alanine, respectively. The leucine codon in position 111 of the human *GRB2* gene was mutagenized to an amber codon by using the QuikChange site-directed mutagenesis kit (Stratagene) to create *GRB2*(Am111). The hemagglutinin tag (YPYDVPDYA) was added to the C termini of the wild-type *E. coli* TyrRS and *Eco*-pBpaRS, whereas the wild-type *GRB2* and *GRB2*(Am) genes were tagged with the Flag sequence (DYKDDDDK) at their C termini. The wild-type and variant *GRB2* and TyrRS genes and the EGF receptor gene were each cloned in the pcDNA4/TO vector (Invitrogen) for expression in the cells. The wild-type *GRB2* gene in the vector was mutagenized using the QuikChange site-directed mutagenesis kit, to generate a series of clones with an amber codon at positions 103–110, 112, 119, 121 and 143. A plasmid carrying nine copies of the amber suppressor tRNA gene has been previously described¹⁶.

Transfection. T-REx-CHO cells (Invitrogen), which constitutively produce the tetracycline repressor, were grown to 90% confluence in a 100-mm dish and then transfected with the pcDNA4/TO plasmids and the suppressor tRNA-expressing plasmid, using the LipofectAMINE 2000 reagent (Invitrogen) in the Gibco Opti-MEMI growth medium (Invitrogen). Four hours after transfection, this medium was replaced by serum-free Gibco D-MEM/F-12 medium (Invitrogen) containing 20 mM HEPES-NaOH (pH 7.2) and 1 µg/ml tetracycline. pBpa was added to this medium at a concentration of 1 mM. The cells were used in experiments after a 12-h incubation.

Western blotting. Cells were lysed in buffer A: 30 mM Tris-HCl buffer (pH 7.4), 10% glycerol, 1% Triton X-100, 5 mM EDTA and 0.05% sodium deoxycholate, with a 1:100 dilution of proteinase

inhibitor cocktail (Nakarai Tesque). The proteins were then fractionated by SDS-PAGE and transferred to PVDF membranes (Millipore). The proteins on the membrane were probed with antibodies and then detected with the ECL plus immunodetection system (Amersham Biosciences). The membranes were stripped in 62.5 mM Tris-HCl buffer (pH 6.8) containing 2% SDS and 0.7% 2-mercaptoethanol for 30 min at 55 °C to prepare for probing with the second antibody. The band intensity was measured with an image analyzer, LAS-1000plus (Fuji Photo Film).

Immunoprecipitation of the Grb2 variants with the EGF receptor and endogenous proteins. For coimmunoprecipitation with the EGF receptor, the cells were treated with EGF, washed twice with ice-cold PBS, pH 7.2, and then lysed in buffer A containing 1 mM orthovanadate. The extracts were incubated at 4 °C for 2 h with affinity gels linked to the anti-Flag M2 antibodies, and the proteins thus trapped on the gels were eluted by the Flag peptide (Sigma). For coimmunoprecipitation with endogenous proteins, the cells were not stimulated with EGF.

Photo-cross-linking of the Grb2 variants with the EGF receptor and endogenous proteins. For photo-cross-linking with the EGF receptor, the cells were treated with EGF and washed as described above. The culture dishes containing these cells in PBS were placed on ice and exposed to 365-nm light by using an 8-W lamp (UVP), at a 2.5-cm distance. Immediately after light exposure, the cell lysate was prepared in buffer A and then treated with the protein tyrosine phosphatase 1B at 0.5 U/μl (Calbiochem) for 30 min at 30 °C. For photo-cross-linking with endogenous proteins, the quiescent cells were washed with PBS and exposed to the light, and then were lysed in buffer A containing 1 mM orthovanadate. The lysate was not treated with phosphatase.

Note: Supplementary information is available on the Nature Methods website.

ACKNOWLEDGMENTS

This work was supported by the RIKEN Structural Genomics/Proteomics Initiative (RSGI), the National Project on Protein Structural and Functional Analyses and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 21 December 2004; accepted 20 January 2005

Published online at <http://www.nature.com/naturemethods/>

- Fields, S. & Song, O. A novel genetic system to detect protein-protein interactions. *Nature* **340**, 245–246 (1989).
- Drees, B.L. Progress and variations in two-hybrid and three-hybrid technologies. *Curr. Opin. Chem. Biol.* **3**, 64–70 (1999).
- MacBeath, G. Protein microarrays and proteomics. *Nat. Genet.* **32**, Suppl., 526–532 (2002).
- Zhu, H. & Snyder, M. Protein chip technology. *Curr. Opin. Chem. Biol.* **7**, 55–63 (2003).
- Brunner, J. New photolabeling and crosslinking methods. *Annu. Rev. Biochem.* **62**, 483–514 (1993).
- Dorman, G. & Prestwich, G.D. Benzophenone photophores in biochemistry. *Biochemistry* **33**, 5661–5673 (1994).
- Gergel, J.R. *et al.* Identification of amino acids in the N-terminal SH2 domain of phospholipase C gamma 1 important in the interaction with epidermal growth factor receptor. *Biochemistry* **33**, 14671–14678 (1994).
- Kage, R., Leeman, S.E., Krause, J.E., Costello, C.E. & Boyd, N.D. Identification of methionine as the site of covalent attachment of a *p*-benzoyl-phenylalanine-containing analogue of substance P on the substance P (NK-1) receptor. *J. Biol. Chem.* **271**, 25797–25800 (1996).
- Kanamori, T., Nishikawa, S., Shin, I., Schultz, P.G. & Endo, T. Probing the environment along the protein import pathways in yeast mitochondria by site-specific photocrosslinking. *Proc. Natl. Acad. Sci. USA* **94**, 485–490 (1997).
- Brunner, J. Use of photocrosslinkers in cell biology. *Trends Cell Biol.* **6**, 154–157 (1996).
- Chin, J.W., Martin, A.B., King, D.S., Wang, L. & Schultz, P.G. Addition of a photocrosslinking amino acid to the genetic code of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **99**, 11020–11024 (2002).
- Chin, J.W. & Schultz, P.G. *In vivo* photocrosslinking with unnatural amino acid mutagenesis. *ChemBiochem* **3**, 1135–1137 (2002).
- Kobayashi, T. *et al.* Structural basis for orthogonal tRNA specificities of tyrosyl-tRNA synthetases for genetic code expansion. *Nat. Struct. Biol.* **10**, 425–432 (2003).
- Drabkin, H.J., Park, H.J. & RajBhandary, U.L. Amber suppression in mammalian cells dependent upon expression of an *Escherichia coli* aminoacyl-tRNA synthetase gene. *Mol. Cell Biol.* **16**, 907–913 (1996).
- Kiga, D. *et al.* An engineered *Escherichia coli* tyrosyl-tRNA synthetase for site-specific incorporation of an unnatural amino acid into proteins in eukaryotic translation and its application in a wheat germ cell-free system. *Proc. Natl. Acad. Sci. USA* **99**, 9715–9720 (2002).
- Sakamoto, K. *et al.* Site-specific incorporation of an unnatural amino acid into proteins in mammalian cells. *Nucleic Acids Res.* **30**, 4692–4699 (2002).
- Chin, J.W. *et al.* An expanded eukaryotic genetic code. *Science* **301**, 964–967 (2003).
- Rozakis-Adcock, M. *et al.* Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature* **360**, 689–692 (1992).
- Buday, L. & Downward, J. Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. *Cell* **73**, 611–620 (1993).
- Rahuel, J. *et al.* Structural basis for the high affinity of amino-aromatic SH2 phosphopeptide ligands. *J. Mol. Biol.* **279**, 1013–1022 (1998).
- Nioche, P. *et al.* Crystal structures of the SH2 domain of Grb2: highlight on the binding of a new high-affinity inhibitor. *J. Mol. Biol.* **315**, 1167–1177 (2002).
- Ogura, K. *et al.* Solution structure of the SH2 domain of Grb2 complexed with the Shc-derived phosphotyrosine-containing peptide. *J. Mol. Biol.* **289**, 439–445 (1999).
- Tzahar, E. *et al.* A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol. Cell Biol.* **16**, 5276–5287 (1996).
- Coker, K.J., Staros, J.V. & Guyer, C.A. A kinase-negative epidermal growth factor receptor that retains the capacity to stimulate DNA synthesis. *Proc. Natl. Acad. Sci. USA* **91**, 6967–6971 (1994).
- Burke, P., Schooler, K. & Wiley, H.S. Regulation of epidermal growth factor receptor signaling by endocytosis and intracellular trafficking. *Mol. Biol. Cell* **12**, 1897–1910 (2001).
- Wang, L., Brock, A., Herberich, B. & Schultz, P.G. Expanding the genetic code of *Escherichia coli*. *Science* **292**, 498–500 (2001).
- Hendrickson, T.L., Crecy-Lagard, V. & Schimmel, P. Incorporation of nonnatural amino acids into proteins. *Annu. Rev. Biochem.* **73**, 147–176 (2004).
- Zhang, Z. *et al.* Selective incorporation of 5-hydroxytryptophan into proteins in mammalian cells. *Proc. Natl. Acad. Sci. USA* **101**, 8882–8887 (2004).
- Rigaut, G. *et al.* A generic protein purification method for protein complex characterization and proteome exploration. *Nat. Biotechnol.* **17**, 1030–1032 (1999).
- Agou, F. *et al.* NEMO trimerizes through its coiled-coil C-terminal domain. *J. Biol. Chem.* **277**, 17464–17475 (2002).
- Chen, H.T. & Hahn, S. Binding of TFIIB to RNA polymerase II: mapping the binding site for the TFIIB zinc ribbon domain within the preinitiation complex. *Mol. Cell* **12**, 437–447 (2003).