Identification of a hypothetical membrane protein interactor of ribosomal phosphoprotein P0

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The ribosomal phosphoprotein P0 of the human malarial parasite *Plasmodium falciparum* (PfP0) has been identified as a protective surface protein. In *Drosophila*, P0 protein functions in the nucleus. The ribosomal function of P0 is mediated at the stalk of the large ribosomal subunit at the GTPase centre, where the elongation factor eEF2 binds. The multiple roles of the P0 protein presumably occur through interactions with other proteins. To identify such interacting protein domains, a yeast two-hybrid screen was carried out. Out of a set of sixty clones isolated, twelve clones that interacted strongly with both PfP0 and the *Saccharomyces cerevisiae* P0 (ScP0) protein were analysed. These belonged to three broad classes: namely (i) ribosomal proteins; (ii) proteins involved in nucleotide binding; and (iii) hypothetical integral membrane proteins. One of the strongest interactors (clone 67B) mapped to the gene YFL034W which codes for a hypothetical integral membrane protein, and is conserved amongst several eukaryotic organisms. The insert of clone 67B was expressed as a recombinant protein, and immunoprecipitaion (IP) reaction with anti-P0 antibodies pulled down this protein along with PfP0 as well as ScP0 protein. Using deletion constructions, the domain of ScP0, which interacted with clone 67B, was mapped to 60–148 amino acids. It is envisaged that the surface localization of P0 protein may be mediated through interactions with putative YFL034W-like proteins in *P. falciparum*.

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1. Introduction

In the eukaryotic large ribosomal subunit, a group of phosphoproteins (P proteins) form a complex and reside at the stalk of the subunit at the GTPase centre (Rich and Steitz 1987; Uchiumi *et al* 1987; Uchiumi and Kominami 1992). These proteins, P0, P1 and P2, possess a conserved 22 amino acid carboxyl-terminal region, and have been characterized earlier through co-precipitation studies using antibodies against this conserved carboxyl-terminal region (Rich and Steitz 1987). Protein P0 is also involved at the eEF2 elongation factor-binding domain, as demonstrated in yeast (Justice *et al* 1999). The P0 protein, and not P1 and P2 proteins, is essential for viability in yeast; and the vital domain of yeast P0 is mapped to 132 amino acids at the carboxy-terminal region (Santos and Ballesta 1994, 1995). Recent studies have revealed other functions of the ribosomal protein P0. In *Drosophila* it has been shown that P0 protein possesses DNA-binding activity and, specifically apurinic/apyramidinic endonuclease activity in the nuclei (Yacoub *et al* 1996). P0 protein in *Drosophila* has also been shown to play a regulatory role in

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Abbreviations used: GST, glutathione-S transferase; IP, immunoprecipitation; ORF, open reading frame; PfP0, *Plasmodium falciparum* phosphoriboprotein P0; ScP0, *Saccharomyces cerevisiae* phosphoriboprotein P0.

modifying gene expression, and show position-effect variegation (Craig and Denlinger 2000). The level of P0 protein is regulated during development, carcinogenesis and apoptosis (Brockstedt *et al* 1998; Frolov and Birchler 1998; Liliensiek *et al* 1998). We have recently demonstrated the presence of the human malarial parasite *Plasmodium falciparum* P0 protein (PfP0) on the surface of the sexual and asexual invasive stages of the parasite (Chatterjee *et al* 2000a). We have shown that specific antibodies raised to different domains of PfP0 can block the invasion of *P. falciparum* merozoites into red blood cells (Goswami *et al* 1997; Chatterjee *et al* 2000a), and confers protection to mice challenged with the lethal 17XL strain of *P. yoelii* (Chatterjee *et al* 2000b). P0-like determinant has been reported on the membrane of mammalian cell lines and tissues earlier (Koren *et al* 1992; Sun *et al* 1996). We have observed that the P0 protein is localized on the surface of other protozoan parasites (*Toxoplasma*), yeast and mammalian cells (Singh *et al* 2002). Recently we have demonstrated the translocation of the *Toxoplasma gondii* P0 (TgP0) protein on the surface of parasite cells by detecting the presence of HA-tag on the surface of *T. gondii* cells transfected with TgP0 gene tagged with the HA-tag (Sehgal *et al* 2003). However, the primary amino-acid sequence of P0 shows neither a signal sequence, nor any significant transmembrane domain(s). Therefore, we envisage that P0 may be transported to the surface through interactions with other proteins. To identify such interactors of P0 protein we carried out a yeast two-hybrid screen using P0 protein as the bait. In this paper we report the preliminary analysis of twelve interactors of P0 identified in this screen. The protein domain of the hypothetical integral membrane protein, YFL034W, showed a strong interaction with P0 genetically, and could be pulled down in an immunoprecipitation reaction. Using deletion clones, we map 60–148 amino acids of the ScP0 protein to be the domain interacting with the YFL034W protein.

2. Materials and methods

2.1 *Cell strains and plasmids*

Yeast two hybrid plasmids, pEG202, pSH18-34, pJG4-5 and the yeast strain EGY48 were obtained from Roger Brent's lab and have been described earlier (Gyuris *et al* 1993). The PfP0 (316 amino acids) was amplified from *l*12 clone (Goswami *et al* 1996), and the yeast P0 (312 amino acids) was amplified from genomic DNA using primers YP0FR1 (5′CTGAATTCATGGGAGGCATTCGTGAAAAG3′) and YP0RX1 (5′CGGCCTCGAGTCATGTTGCACTTC3′). These were ligated into pEG202 in frame resulting in plasmids pEGPfP0 and pEGScP0 respectively. The deletion clones of ScP0 were subcloned using appropriate primers in reading frame with the pEG202 plasmid. ScP1*a* gene was amplified using (5′CTGAATTCATGTCTACTGAATCCGC3′) and (5′ CTCTCGAGCTAATCAAATAAACCGAAAC3′) primers, and cloned in the pJG4-5 vector in the correct reading frame. The library used for screening was from yeast (gift from Roger Brent). It was made by partial restriction of yeast genomic DNA (strain S288c) using *Alu*I and *Hae*III restriction enzymes, methylase protection of the *Eco*RI sites, insertion of *Eco*RI linkers, restriction with *Eco*RI, isolation of 800–4000 bp fragments, ligation in pJG4-5 vector and transformation into *Escherichia coli* sure cells. 3×10^6 original transformants were collected and grown to amplify the plasmid DNA (pJG4-5 yeast library plasmid DNA).

2.2 *Yeast two-hybrid screening*

The screen was performed as detailed elsewhere (Gyuris *et al* 1993). Briefly, the yeast strain EGY48 (MAT*a*, *his3*, *trp1*, *ura3*-52, *leu2 :: pLEU2-LexAop6*) was pre-transformed by the lithium acetate method, with the bait pEGPfP0 and the lacZ reporter pSH18-34, which contains 8 LexA operator-binding sites (LexAop). These transformants were tested for the lack of growth on Leu^- plates. These cells were then transformed with 2 μg pJG4-5 yeast-library plasmid DNA and grown on glucose Ura^- His^- Trp^- plates. About $0{\cdot}5 \times 10^6$ transformants were scraped, pooled and amplified in the same liquid medium. About $0{\cdot}5 \times 10^5$ of these colonies were then plated onto galactose containing minimal medium plates. Approximately 350 Leu^+ colonies were replica-plated onto *b*-galactosidase assay plates containing either galactose or dextrose as the carbon source. Sixty Leu^+ colonies that grew and demonstrated *b*-galactosidase activity on galactose, but not on dextrose, were further characterized. Based on *b*-galactosidase activity, twelve clones with comparatively higher activity were taken up for further analysis. The recombinant pJG4-5 plasmid from each of these was isolated by transformation of KC8 *E. coli* cells on Trp^- ampicillin plates, and the rescued plasmid was sequenced using an automated sequencer. Each of the twelve pJG4-5 recombinant plasmid DNA was then reintroduced with either of the two bait plasmids, pEGPfP0 or pEGScP0, along with the reporter plasmid pSH18-34 into EGY48 yeast cells to revalidate the interactions. Control EGY48 cells containing bait vector pEG202 (without P0) and the reporter plasmid pSH18-34, were transformed with each of the interactor pJG4-5 plasmids, but no growth was observed on Leu^- plates.

2.3 *b-galactosidase assay*

Filter-lift and quantitative *b*-galactosidase measurements were carried out with yeast liquid cultures, using the procedure described by the Clonetech. Typically, 0·1 ml of

cells were harvested in 200 µl of phosphate buffer, pH 7·0, permeabilized by liquid nitrogen treatment, and 0·8 mg of the substrate o-nitrophenyl-*b*-D-galactopyranoside (ONPG) was added to start the reaction. The specific activity was measured as described earlier (Miller 1972).

2.4 *Expression of GST-67, ScP0 and PfP0 proteins and immunoprecipitation assay*

GST-reporter based vectors were used for the expression of PfP0, ScP0 and the interactor clone 67B protein domains (figure 2, panel I). The expression of PfP0C containing the carboxy-terminal 256 amino acid domain of PfP0 has already been described earlier (Chatterjee *et al* 2000a). The full-length yeast P0 (ScP0) and the 1·1 kb open reading frame (ORF) obtained from clone 67, were also expressed as GST fusion proteins using the vector pGEX-4T-1 and pGEX-2T, respectively (Amersham Biosciences, UK). The recombinant *E. coli* cells containing PfP0C, ScP0 and yeast 67B domains were grown for 2 h at 37°C, induced with 0·5 mM iso-propyl-*b*-D-thiogalactopyranoside (IPTG) and grown for another 2 h. The cultures were then harvested, suspended in phosphate buffered saline (PBS) containing 2% Triton X 100, and sonicated with a Branson sonifier. PfP0C and 67B fusion proteins were purified by electroelution using protocol described earlier (Chatterjee *et al* 2000a). ScP0 fusion protein was affinity-purified using GST-Sepharose 4B beads as per manufacturer's protocol (Amersham Biosciences, UK). The purified proteins were run on a 10% denaturing SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue to show the fusion proteins (figure 2, panel IIB). Full length PfP0 protein was also expressed in the vector pFL37 under the control of ScP0 regulatory sequences in W303dGP0 knockout strain of ScP0 (Rodriguez-Gabriel *et al* 2000). The plasmid pFL37 and the W303dGP0 strain were kind gifts of J P G Ballesta. IP assays were carried out using protocol as described earlier (Goswami *et al* 1997). Purified polyclonal rabbit serum raised against PfP0C protein (Chatterjee *et al* 2000a) was used for the IP studies of the GST-fusion proteins. Specific monoclonal antibodies were raised in our laboratory against PfP0 protein (K Rajeshwari, K Patel, M Mehtha and S Sharma, unpublished results) and a pool of three such mAbs were used for the IP reaction of PfP0 protein expressed in yeast.

3. Results and discussion

The yeast two-hybrid interaction screen was performed using the *P. falciparum* P0 protein as bait, and yeast genomic partial digest expression library as the source for interactor genes. From a set of sixty Leu^+ interactor-pJG4-5 clones, twelve independent clones with comparative high *b*-galactosidase activity were selected for analysis. These twelve inserts were also radioactively labelled and used as probes to determine the multiplicity of representation of each of these clones. Table 1 shows the *b*-galactosidase activity and the frequency of these clones in the set of sixty clones. Clone No. H2S represented 15% of this set, while 7% and 5% of the clones belonged to the clones 77T and 71T, respectively. The remaining nine clones were found to be unique.

Table 1. Interactor protein genes and *b*-galactosidase activity of the clones.

Clone No.	Gene/protein name in *S. cerevisiae*	No. of transformants (out of 60)	*b* galactosidase activity (Miller units)[a] PfP0[b]	*b* galactosidase activity (Miller units)[a] ScP0[b]	Filter lift assay with PfP0[b]
64B	RPL7A/YGL076C	1	3·4	2201	++±
48A	TUB2/YFL037W	1	6·0	319	ND[c]
77T	ADH1/YOL086C	4(7%)	1·8	521	+±
38T	GAL83Y/YER027C/	1	2·9	320	+±
100A	TY4B GAG/POL/ YHL009W-B	1	133·6	2042	++++++++
66A	BRR2/YER172C	1	1·4	717	++±
60A	YIL177C	1	13·2	600	ND
H2S	YPL260W	9(15%)	5·0	2846	+++
67B	YFL034W	1	121·2	467	+++++
60T	YDR387C	1	2·2	28	++±
71T	YOR296W	3(5%)	1·1	303	±
58T	YJL207C	1	0·1	486	±

[a]Miller units, based on substrate ONPG, were calculated as described in Miller (1972).
[b]Respective DNA binding domains.
[c]ND, Not determined.

Ideally the 2-hybrid screen should have been performed with pJG4-5 containing a cDNA library of *P. falciparum*. However, because of the inaccessibility of such a library, the instability of recombinant clones of *P. falciparum* (with an average of about 80% AT rich residues), and the codon-bias problems, we decided to use the yeast expression library for the two-hybrid screen. The ribosomal P0 proteins are well conserved across eukaryotic organisms, and *P. falciparum* P0 protein is closely related to yeast P0 (figure 1). The antibodies against PfP0 protein cross-reacts with yeast P0, and yeast P0 is also found on the surface of yeast spheroplasts (Singh *et al* 2002). Using the conditional yeast P0 null strain W303dGP0 (Rodriguez-Gabriel *et al* 2000), we have recently shown that PfP0 can complement yeast P0 (K Aruna, J P Ballesta and S Sharma, unpublished results). It was envisaged that the *S. cerevisiae* is a system close to *P. falciparum* and that for most yeast interactors identified, there would be homologues in *Plasmodium*, which can then be studied. However, to test whether these 2-hybrid interactions were also meaningful in yeast, the yeast P0 DNA was also cloned in the bait-vector, and the twelve interactor clones were tested. Each of these transformants grew on Leu^- selection, validating the interactions. The control cells containing just the bait vector (pEG202, without any P0 insert), showed no growth on Leu^- plates. These cells transformed with either the pJG4-5 plasmid alone, or with plasmid containing each of the twelve interactor-yeast gene insert, also showed no growth on Leu^- plates.

The filter lift assay was used to assess the *b*-galactosidase activity. With PfP0 the colour developed over several minutes, and could be scored (table 1). However, when ScP0 was used as the bait, the activity was much higher, and the blue colour developed simultaneously within a few seconds in the filter-lift assay, and the differences between different clones could not be scored. In order to assess the *b*-galactosidase activity quantitatively, ONPG assays were carried out with yeast transformants containing PfP0 as well as ScP0 (table 1). It is observed that the activities obtained with ScP0 were much higher

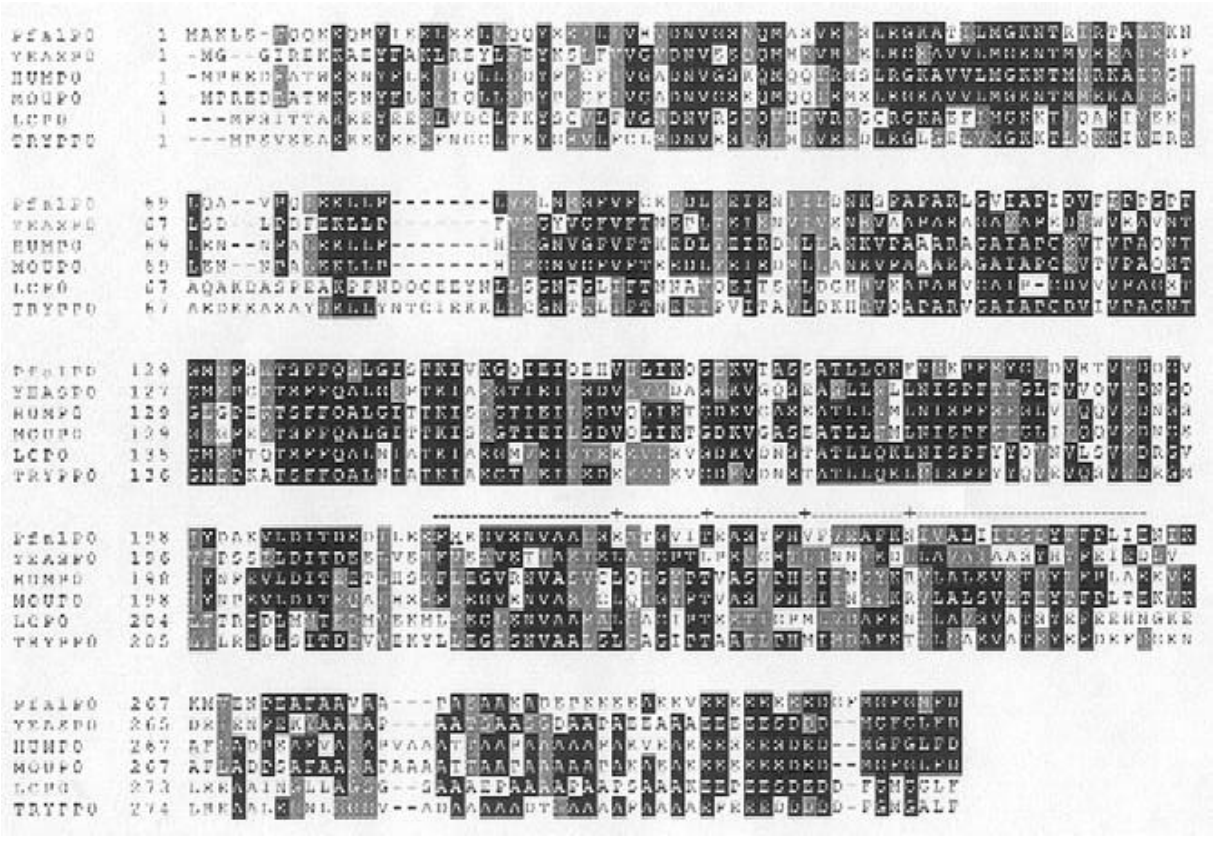

Figure 1. Box shading on EBI Clustalw alignment of P0 sequences from different organisms. PfalP0, *Plasmodium falciparum* P0 (U56663); YEASP0, *Saccharomyces cerevisiae* P0 (P05317); HUMP0, *Homo sapiens* P0 (P05388); MOUP0, *Mus musculus* P0 (P14869); LCP0, *Leishmania chagasi* P0 (P39096); TRYPP0, *Trypanosoma cruzi* P0 (P26796). Identical residues are shaded black and similar residues are shaded gray. The dotted line shows the ScP1 binding domain, and the '+' sign denotes the leucine residues found in ScP0, constituting the putative leucine zipper as predicted by Lalioti *et al* (2002).

than those containing PfP0. Clone No. 100A exhibited high *b*-galactosidase activity for both the PfP0 and ScP0. The response of the rest were mixed. In case of PfP0, clone No. 67B showed high *b*-galactosidase activity, while clone Nos 58T and 71T showed the least activities.

Table 2 shows the identity of these twelve yeast gene interactors of PfP0. The identifiable proteins were (i) ribosomal protein RPL7A; (ii) *b*-tubulin protein TUB2; (iii) alcohol dehydrogenase; (iv) glucose repression protein Gal83Y; (v) TY4B polyprotein; and (vi) RNA helicase protein involved in m-RNA splicing. About 50% of the interactor clones identified were found to code for hypothetical proteins, with postulated transmembrane domains.

Through cross-linking and binding studies in brine shrimp, rat and yeast, it has been established earlier that P0, P1 and P2 phosphoribosmal proteins form a pentameric complex $(P1)_2.P0.(P2)_2$ (Uchiumi and Kominami 1992). Recent *in vitro* binding analysis has shown that only P1 protein binds directly to P0 protein, and only then P2 protein may be able to bind (Zurdo *et al* 2000). Indeed P1 has been observed to be the only genetic interactor of P0 protein in yeast (Lalioti *et al* 2002). However, P1 clone was not present amongst the sixty interactor clones isolated, as determined through dot-blot hybridization using PCR amplified radioactive probes (data not shown). Protein P1 is postulated to interact with 212–262 amino acids at the carboxy-terminal domain of ScP0, and this domain is characterized with a putative leucine zipper structure (Lalioti *et al* 2002). PfP0 shows distinct differences from ScP0 in this carboxy-terminal domain, and does not contain this putative leucine zipper motif (figure 1). Thus the failure to identify P1 protein in this screen could be due to lack of interaction of ScP1 with PfP0. To test this hypothesis, yeast P1 was cloned in the TRAP pJG4-5 vector and interaction of ScP0 and PfP0 was tested. Yeast contains two P1 genes, *ScP1a* and *ScP1b*, amongst which *ScP1a* was the clone chosen for this experiment, as it has been demonstrated that *ScP1a* is a better interactor of ScP0 (Lalioti *et al* 2002). It was observed that while *ScP1a* interacted with ScP0 (growth on Leu^- plates and

Table 2. Identity of the interactor proteins.

Clone	Gene in *S. cerevisiae*	Identity of proteins	Homologues	Predicted homologues in *P. falciparum*[a]		Established features in *S. cereivisiae*[b]	
				Gene	Score	Interactors	Knockouts
64B	YGL076C	60S ribosomal protein RPL7	Conserved	Chr3. phat_97	6e-53	Not known	Viable
48A	YFL037W	Beta tubulin	Conserved	Chr10.phat_104	0·0	Many	Lethal
77T	YOL086C	Alcohol dehydrogenase	Conserved	Not found	–	TUB1	Viable
38T	YER027C	Glucose repression protein	Conserved	Chr6.glm40	0·24	SNF4 and SNF1	Viable
100A	YHL009W-B	TY4B gag pol protein	With YJL114W (gag protein)	Chr11.phat_376	5e-12	YJL114W interacts with SNF4	Viable
66A	YER172C	RNA-Helicase	Conserved	Chr14.phat_364	6e-94	SNP 1 and CUS 1	Lethal
H2S	YPL260W	HMP[c] (1 TM)[d]	Conserved	Chr14.phat_599	3·9	TID 3	Viable
60A	YIL177C	HMP (4 TM)	Possess domain homology with helicases	Chr14.phat_647	0·019	Not known	Not reported
67B	YFL034W	HMP (7 TM)	Conserved	Chr12.gen_504	1e-40	Not known	Viable
60T	YDR387C	HMP (12 TM)	Domain homology with hexose transporter family proteins	Chr2. phat_64	7e-18	Not known	Viable
71T	YOR296W	HMP (2 TM)	Not known	Chr13.glm_382	0·79	Not known	Viable
58T	YJL207C	HMP (9 TM)	Not known	Chr9.gen_38	0·002	Not known	Viable

[a]The URL for this is http://www.plasmodb.org.
[b]Interaction reports and systematic deletions are from *Saccharomyces* Genome Database. The URL for this is http://genome-www.stanford.edu/Saccharomyces/.
[c]Hypothetical membrane protein.
[d]All transmembrane (TM) domain predictions are made by the use of Tmpred program. The URL for this program is http://www.ch.embnet.org/software/TMPRED_form.html.

blue colour on X-gal), it did not grow on Leu$^-$ plates when co-transfected with PfP0 (data not shown), validating the postulate that the failure to identify P1 was due to the lack of interaction between ScP1 with PfP0.

Of the 12 interactors, the identification of another ribosomal protein (RPL7A) (Mizuta *et al* 1995) corroborates the ribosomal property of the P0 protein. The human homologue of this protein has been shown to possess 28S rRNA binding domain (von Mikecz *et al* 1999). The identification of RNA helicases also indicates domains involved in RNA binding, which P0 protein possesses (Rodriguez-Gabriel *et al* 2000). In the ribosome, P0 protein plays a role at the GTPase centre, in the binding of elongation factor eEF2 (Rich and Steitz 1987). Since certain GTP-binding pockets are conserved in tubulins, dehydrogenases and elongation factor (Maessen *et al* 1993), it is possible that some of these other interactors identified in our screen recognize such a domain. However, such a postulate has to be tested for each of these interactors. The most interesting set of proteins, the hypothetical integral membrane proteins (table 2), provides a possible explanation regarding the surface translocation of P0. One of these showed domain homology to helicase, and one to hexose transporter family proteins, but the rest showed no significant domain homology to any other known protein.

At present much of the genome data of *P. falciparum* is available, and therefore a BLAST search was made for each of these yeast interactor genes, and the results are summarized in table 2. Of these, *b*-tubulin (YFL037W), RNA helicase (YER172C), the ribosomal protein (YGL076C) and the *YDR387C* gene with domain homology to the hexose transporter gene, showed significant scores for *P. falciparum*. In addition, YFL034W, the hypothetical *trans*-membrane protein of yeast (clone No. 67B) also showed a very conserved predicted homologue (chr12.gen_504) on chromosome 12 of *P. falciparum* (table 2). In *P. falciparum*, this 1082 amino acid ORF has been annotated as PFL0295c, a protein with 36% identity to 64% of a putative *Mus musculus* protein (Accession No. AK014571). This hypothetical *trans*-membrane protein is conserved across *Arabidopsis*, human and *C. elegans* as well. The TMPRED analysis predicted a 4–7 *trans*-membrane structure for the YFL034W yeast protein, and 4–5 *trans*-membrane domains for the *P. falciparum* protein.

To test whether the hypothetical integral membrane protein YFL034W binds to the P0 protein, clone 67B was expressed in *E. coli*, and IP experiment was carried out with PfP0 and ScP0 (figure 2). Attempts to express these proteins as non-GST-fusion proteins in *E. coli* were not successful, and the only stable expression for each of these proteins was obtained as GST-fusions. The domains of PfP0, ScP0 and YFL034W (clone 67B) expressed as GST-fusion proteins are shown schematically in figure 2, panel I. The purified GST-fusion proteins, at molecular weights of 68 kDa (GST67B); 51 kDa (PfP0C) and 58 kDa (GST-ScP0), are shown in figure 2, (panel IIA). The anti-PfP0C antibody was also found to cross-react specifically with ScP0, as shown through an immunoblot analysis (figure 2, panel IIB). The purified antibody, which was used for the IP reaction, was tested on a dot blot to ensure that there was no reactivity with either the GST domain or with the 67B fusion protein (figure 2, panel IIC). The immunoprecipitate was resolved on a 10% SDS-PAGE, blotted on nitrocellulose and probed with anti-GST antibodies (figure 2, panel IID). Specific pull-down of 67B, along with each of PfP0 and ScP0 (lanes 3 and 5) was observed. IPs under identical conditions, but without P0 protein, did not show any pull down protein (lane 1), as well as pre-immune antibody did not show specific band (lane 4). Since the cross-reactivity of the anti-PfP0 antibody to ScP0 was weak, the immunoprecipitation of 67B with ScP0 is less specific and weaker (lane 5).

Since both the P0 and 67B expressed proteins were GST-fusion proteins, it was possible that the pull down occurred due to the interaction of GST domains. Attempts to cleave the GST from the fusion proteins were not successful. In order to rule out the involvement of the GST domain, full length PfP0 expressed in yeast was used (figure 3). The crude protein extract of the ScP0 knock-out strain W303dGP0, expressing PfP0 protein under the ScP0 regulatory regions (K Aruna, J P Ballesta and S Sharma, unpublished results) was used. The presence of PfP0 protein in the crude extract of the transfected W303dGP0 strain is detected through immunoblotting and screening with mAbs against PfP0. These monoclonal antibodies reacted very specifically only with the 38 kDa PfP0 protein in Sc crude extract, and did not react with the purified GST-67B protein. Panels C-E show the pull down of 70 kDa GST-67B protein in the presence of the yeast crude extract containing the PfP0 protein. Since the secondary antibodies used in the immunoblot were anti-mouse IgG, the IgG molecules used for IP also light up in the blot (figure 3E). An immunoblot of IP carried out with yeast crude extract containing the PfP0 protein, in the absence of 67B protein showed a profile identical to lane 2 in figure 3E, while the control IP reaction with the 67B protein alone, in the absence of yeast crude extract containing the PfP0 protein, does not pull down 67B (lane 1, figure 3E). These results clearly show that there is specific biochemical interaction between P0 and the 67B domain of YFL034W protein.

To examine which domain of P0 protein was interacting with clone 67B, various deletion clones of ScP0 were constructed (figure 4). The results show that the binding domain is present within the K construct (180–600 bp), since it grew on Leu$^-$, and showed blue colonies in X-gal plates. The *b*-galactosidase activities of the full length

ScP0 and construct K, cotransfected with clone 67, were measured to be 450 and 771 Miller units, respectively. Since 444–600 bp construct C did not grow on Leu$^-$ and therefore do not show any interaction, it might be deduced that the interaction domain of ScP0 resides within 180–444 bp (60 to 148 amino acid). To demonstrate that the lack of interaction of construct C, and some of the others, was not due to lack of expression of bait-fusion protein, an immunoblot analysis was carried out using the cross-reactive polyclonal anti-PfP0 sera (figure 5) for various constructs. The DNA-binding domain codes for a 21 kDa protein, and therefore the fusion proteins for full-length ScP0, B, K, C and H are expected to be 56, 36, 37, 26 and 27 kDa respectively. The fusion proteins B and K are of sizes similar to the endogenous ScP0 (36 kDa), which also lights up with the antibodies. However, the

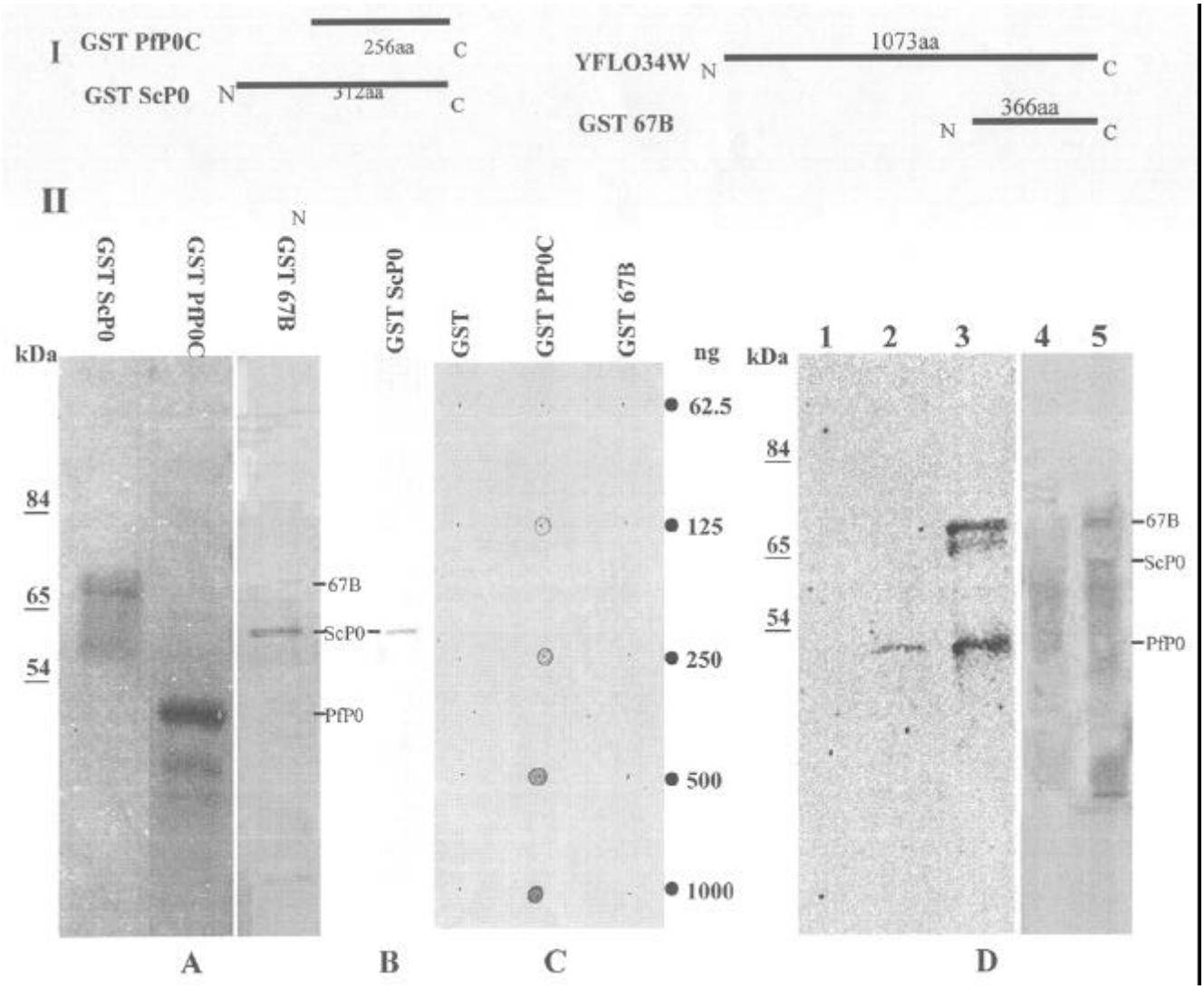

Figure 2. Immunoprecipitation (IP) of clone 67B protein with each of PfP0 and ScP0 proteins. *Panel I*: The different domains of PfP0, ScP0 (full length) and YFL034W, each expressed as a GST-fusion protein, are schematically shown. *Panel II*: (**A**) Purified proteins were resolved on 10% SDS-PAGE followed by Coomassie staining. (**B**) Immunoblot of GSTScP0 protein with anti-PfP0C antibodies (1 : 250 dilution). (**C**) Dot-blot of GST, GSTPfP0 and GST67B probed with anti-PfP0C antibodies. (**D**) Immunoprecipitated proteins run on 10% SDS-PAGE, immunoblotted and probed with anti-GST antibodies. Typically, IP was carried out by mixing of about 20–40 µg of either GST-PfP0 (lanes 2, 3) or GST-ScP0 (lanes 4, 5) with the same amount of 67B protein at 4°C overnight, followed by the addition of 1 : 250 PfP0 polyclonal antibodies (25°C, 4–8 h); followed by incubation with Protein A Sepharose beads (25°C, 1–2 h). The beads were washed, boiled and loaded on SDS-PAGE, followed by Coomassie stain and immunoblotting. Lane 1, 67B protein alone (40 µg); lane 2, PfP0 alone (5 µg); lane 3, 67B (40 µg) and PfP0 (40 µg); lanes 4 and 5, 67B (40 µg) and ScP0 (40 µg). Lane 4 shows the results with pre-immune sera used for IP.

signals for the proteins of constructs K and B at 36 and 37 kDa appear to be more intense compared to the endogenous ScP0 in the full-length ScP0 construct, indicating expression of the bait-fusion proteins (figure 5 A). The amount of fusion protein produced in K appeared to be comparable to B. There is also considerable expression of fusion proteins in constructs C and H (figure 5B). Therefore, the lack of interaction for domains B, C and H does not appear to be due to lack of expression of the bait-fusion protein. Thus the interactive domain of ScP0 for YFL034W possibly lies in the region 60-148 amino acid.

The structural components of the translocation region of the large ribosomal subunit have been studied extensively (Joseph 2003). The prokaryotic and eukaryotic stalk is formed by the pentameric complex L10-$(L7/L12)_2$ and $(P1)_2$.P0.$(P2)_2$, respectively (Uchiumi and Kominami 1992; Bocharov *et al* 1998). In yeast the RNA binding domain and the elongation factor binding domains are postulated to be in the 1–100 amino acid and 110–150 amino acid domains, respectively (Rodriguez-Gabriel *et al* 2000). The eukaryotic protein P0 is larger than its bacterial counterpart protein L10, with a carboxy-terminal extension of about 100 amino acids (Shimmin *et al* 1989). This additional sequence is of vital importance to the organism, as it has been shown that yeast cells do not survive a deletion of 132 carboxy-terminal amino acids (Santos and Ballesta 1994). The P1/P2 binding site has been mapped to 212–262 amino acids, but since the cells are fairly viable in the double knockouts of P1 and P2 (Santos and Ballesta 1995), this region possibly plays other important role(s). Ribosomes are primitive structures in evolution, and many extra-ribosomal properties

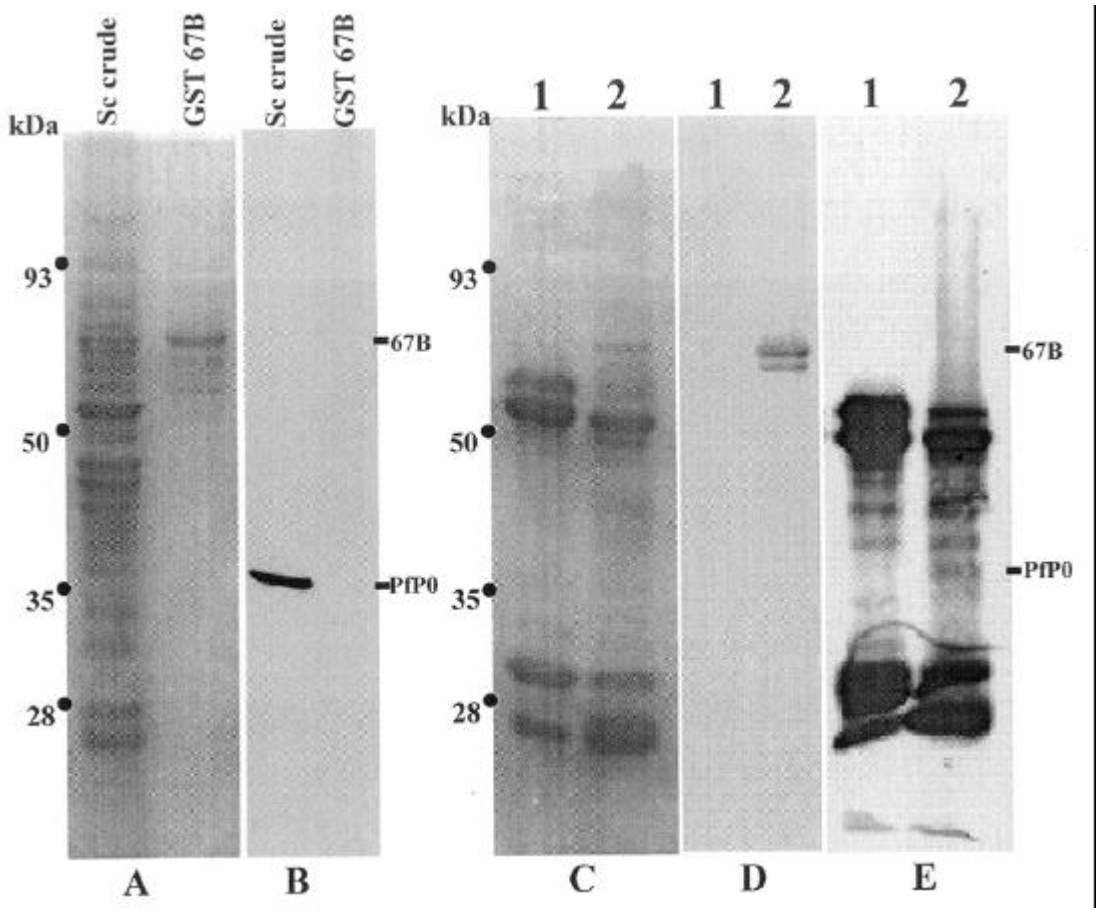

Figure 3. Full length PfP0 protein was expressed in the yeast knockout strain W303dGP0, using the plasmid pFL37 (Rodriguez-Gabriel *et al* 2000). (**A**) About 100 μg of total crude extract from yeast W303dGP0, transfected with PfP0 (lane 1), and 3 μg of purified protein GST67B (lane 2), resolved on 10% SDS-PAGE followed by Coomassie staining. (**B**) The same gel blot treated with a pool of three anti-PfP0 mAbs (culture supernatant). (**C**, **D** and **E**) Identical sets of samples after IP using about 80 μg of purified mAb IgG (protocol same as in figure 2). (**C**) Coomassie stain. (**D** and **E**) Immunoblots probed with D: anti-GST antibodies. (**E**) Anti-PfP0 mAbs, respectively. Lane 1, 35 μg of 67B protein alone; lane 2, 35 μg of 67B protein in the presence of 5 mg of yeast crude extract expressing PfP0.

of ribosomal proteins have been documented earlier (Wool 1996; Tchorzewski *et al* 1999). The DNA binding role and regulatory role of the P0 protein in development and apoptosis (Yacoub *et al* 1996; Brockstedt *et al* 1998; Frolov and Birchler 1998; Liliensiek *et al* 1998; Craig and Denlinger 2000) are indicators of such multiple roles in the ribosomes, nucleus, as well as on the cell surface.

Nothing is known about the mechanism of translocation of the P0 protein to the surface of the cell. Surface localization of P0 is not limited to parasite cells. We have reported surface localization of P0 in parasite, yeast and mammalian cells (Singh *et al* 2002). Surface localization of human P0 on certain mammalian cell lines, as well as on activated T cells have been reported earlier (Koren *et al* 1992; Hirohata and Nakanishi 2001). In these studies, as well as in our work (Goswami *et al* 1997; Chatterjee *et al* 2000a,b; Singh *et al* 2002), the localization was established using antibodies, and has been subject to the question of cross-reactive epitopes belonging to protein(s) other than the ribosomal P0 protein. Using transfected *T. gondii* cells expressing tagged TgP0 protein, we have recently demonstrated that it is indeed the TgP0 protein, which translocates to the cell surface (Sehgal *et al* 2003). Internalization of anti-P0 antibody binding to the human P0 protein has also been documented in live hepatocyte cells (Koscec *et al* 1997), and surface-localized P0 is implicated in Fas-L induced apoptosis (Zampieri *et al* 2001). However, the native function of surface-localized P0 is yet to be elucidated.

The yeast two-hybrid assay reported in this paper is far from a saturation study. In a large-scale proteomic study in yeast, the ScP0 protein has been shown to complex with 13 different proteins, which comprise of other ribosomal proteins, DNA/RNA binding proteins, hypothetical membrane proteins and hexose transporters (Ho *et al* 2002). Although none of these is identical to the twelve genes identified in our yeast two-hybrid screen, the above categories are similar. The lack of identification of YFL034W in the proteomic screen may be due to *in vitro* instability or low abundance of the complex. No functional domain is reported in a BLAST search using this conserved hypothetical *trans*-membrane protein. The interaction with P0 reported in this paper appears to be the first functional attribute to YFL034W. Further, studies are needed to understand the functions of YFL034W, which may also help in understanding the function of the surface-localized P0 protein.

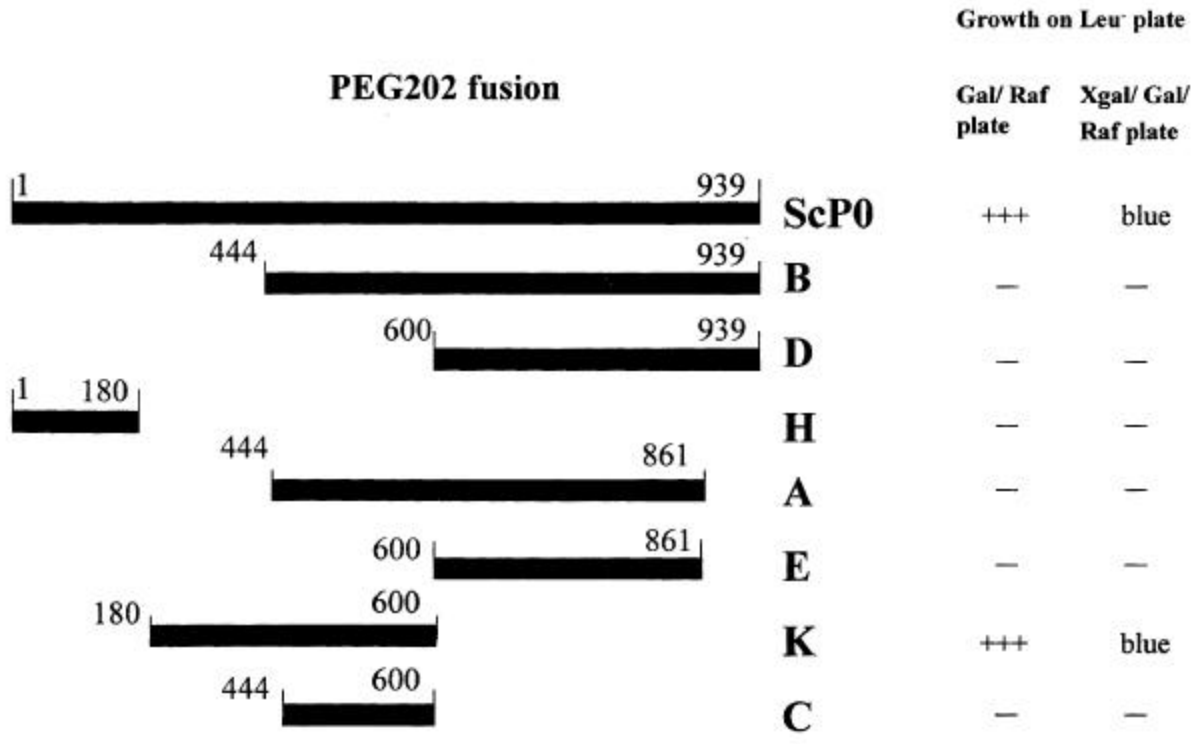

Figure 4. Viability and reporter assay expression with various deletions constructs of ScP0 and clone 67B on U^- T^- H^- L^--gal/raf and U^- T^- H^- L^- X-gal gal/raf plates. Each truncated ScP0 in pEG202 vector and clone 67B in pJG4-5 vector were co-introduced into yeast reporter strain EGY48[pSH18-34]. Numbers on bars indicate the base pair numbers pertaining to the deletion clones of ScP0.

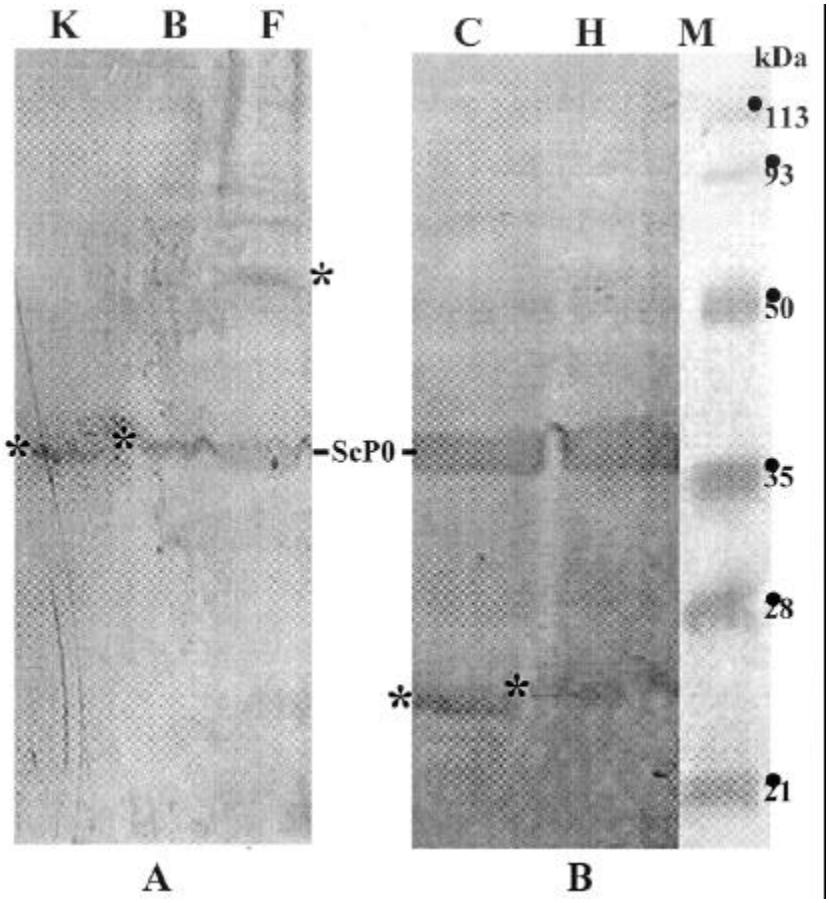

Figure 5. Immunoblot of extracts from yeast cells transformed with the full-length ScP0 or various truncated ScP0 in pEG202 vector. About 100 µg of total crude extract from such transformed yeast cells were resolved on a 10% SDS-PAGE, immunoblotted and probed with anti-PfP0C antibodies (1 : 100 dilution). Lanes K, B, C, H represent the corresponding deletion constructs as shown in figure 4; lanes F and M represent full-length ScP0 and molecular weight markers, respectively. The asterisks show the DNA-binding domain-fusion proteins for each construct.

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