Identification of a Putative Sordarin Binding Site in *Candida albicans* Elongation Factor 2 by Photoaffinity Labeling*

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*Candida albicans* EF-2 binds sordarin to a single class of binding sites with $K_d = 1.26 \mu M$. Equimolar mixtures of EF-2 and ribosomes, in the presence of a non-hydrolyzable GTP analog, reveal two classes of high affinity sordarin binding sites with $K_d = 0.7$ and $41.5 \text{ nM}$, probably due to the existence of two ribosome populations. Photoaffinity labeling of *C. albicans* EF-2 in the absence of ribosomes has been performed with $[^{14}\text{C}]\text{GM258383}$, a photoactivatable sordarin derivative. Labeling is saturable and can be considered specific, because it can be prevented with another sordarin analog. The fragment Glu$^{224}$–Lys$^{322}$ has been identified as the modified peptide within the EF-2 sequence, Lys$^{228}$ being the residue to which the photoprobe was linked. This fragment is included within the G$^\text{G}$-subdomain of EF-2. These results are discussed in the light of the high sordarin specificity toward fungal systems.

The natural product sordarin and its semisynthetic derivatives constitute a selective class of inhibitors of protein synthesis in fungi. Their potential as systemic antifungal agents has been evidenced by their broad spectrum and in *vivo* therapeutic efficiency (1, 2; see Ref. 3 for a recent review). Among all the processes included in protein synthesis, ribosomal translocation is the step impaired by sordarin (4). This molecule binds to EF-2 itself, although such binding is greatly favored by the presence of ribosomes (5). On the other hand, mutations leading to sordarin resistance have been found both in EF-2 and in the yeast ribosomal protein rpP0 (6, 7). Hence, the EF-2-ribosome complex is proposed as the functional target of sordarin antifungals.

Translocation has been thoroughly studied in prokaryotes and a good picture of the global mechanism at molecular level is starting to emerge. Key aspects in such progress have been the elucidation of ribosome structure at good resolution (8) and detailed crystallographic studies of EF-G, the prokaryotic homolog of EF-2 (9, 10). Also, visualization of the ribosome:EF-G complex by cryo-electron microscopy (11), as well as other functional studies (12, 13), have led to a better understanding of the process (see Ref. 14 for a review).

EF-G, the protein that promotes ribosomal translocation in prokaryotes, is a tadpole-like molecule organized into five domains. The globular domain I is responsible for GTP binding and hydrolysis. It contains several structural motifs characteristic of the G-protein superfamily (indeed, it is referred to as “G-domain”) plus an extra insert called the G$^\text{G}$-subdomain. The role of the latter remains unknown, although it has been suggested to act as a nucleotide-exchange factor (9). Interaction of the G-domain with the ribosome triggers GTP hydrolysis, and the energy released is transformed into mechanical movement. Eventually, domain IV (a fibrous domain at the other end of the molecule) moves away, stretching the EF-G shape and literally pushing the newly formed peptidyl-tRNA from the A to the P ribosomal site (15). It is still unclear how energy is transformed into movement, although it seems to be related to rearrangements within both EF-G and the ribosome that allow the complex to act as a molecular ratchet (15, 16).

The eukaryotic system has not been so deeply studied. However, it seems to be more sophisticated, as demonstrated by the larger number of proteins constituting the eukaryotic ribosome and by the ability to regulate EF-2 function by specific kinases (17) and ADP-ribosylation (18). Nevertheless, the general features of the process are assumed to be the same. In coherence with this, there is a substantial degree of homology between bacterial EF-G and eukaryotic EF-2. The more relevant differences are the greater length of EF-2 and the regulatory mechanisms depicted above. Likewise, it is remarkable that the G$^\text{G}$-subdomain has been replaced in EF-2 by another insert termed the G$^\text{G}$-subdomain, which is 15–30 residues longer, with a position that is displaced beyond the sequence, and shows no homology with the prokaryotic G$^\text{G}$-subdomain (19). On the other hand, EF-2 is a highly conserved protein within the entire eukaryotic kingdom. This fact makes especially striking the existence of EF-2 inhibitors such as sordarin, which exclusively impair fungal and not bacterial, mammalian, or plant protein synthesis machinery with the added capacity to discriminate between closely related fungal species (6, 20, 21).

Identification of the residues involved in sordarin binding to EF-2 might help to explain such selectivity. For this reason, we have performed photoaffinity labeling studies of EF-2 from the pathogenic fungus *Candida albicans*, using a radiolabeled sordarin derivative.

**EXPERIMENTAL PROCEDURES**

Elongation factor 2 and salt-washed ribosomes were isolated from *C. albicans* 2005E as previously described (5). EF-2 concentration was determined spectrophotometrically using $A_{260} = 67310 \text{ M}^{-1} \text{cm}^{-1}$ as deduced from its amino acid sequence (Swiss-Prot O13430). Ribosome concentration was also calculated spectrophotometrically assuming one A$_{260}$ unit corresponds to 18 pmol of ribosomes (22). $[^{14}\text{C}]\text{GM258383}$ (1.8 GBq/mmol) and $[^{3}\text{H}]$sordarin (180 GBq/mmoll) were prepared by the Isotope Chemistry Group at GlaxoSmithKline Medicines Research Center (Stevenage, United Kingdom). Non-radiolabeled sordarin derivatives were prepared by the Medicinal Chemistry Unit at GlaxoSmithKline S.A. (Tres Cantos, Spain). Sephadex G-25 (PD-10 columns) was from Amersham Pharmacia Biotech (Uppsala, Sweden). All other chemicals were from Sigma Chemical Co.

**Binding Assays**—Binding of $[^{3}\text{H}]$sordarin to EF-2 alone or to equimo-
lar mixtures of EF-2 and ribosomes was studied by equilibrium dialysis using microvolumetric dialysis capsules (Celul Sep, San Antonio, TX). The two chambers of each capsule were separated by dialysis membrane with a cutoff of 6 kDa. One chamber was filled with 150 μl of [3H]sordarin at appropriate concentration in 25 mM Heps-KOH, pH 7.4, 85 mM potassium acetate, 4 mM magnesium acetate, and 1.5 mM N-dithiothreitol. The other chamber was filled with the same solution containing either 3.6 μM EF-2 or 333 mM EF-2, 333 mM ribosomes, and 33.3 μM Gpp(NH)p. Samples were incubated overnight at 30 °C under rotary shaking. Finally, duplicate 50-μl aliquots were withdrawn from each chamber, and their radioactivity was measured. Free sordarin was calculated from radioactivity values of the first capsule chamber, whereas bound sordarin was calculated by subtracting the latter from each corresponding value of the second capsule chamber.

Photoaffinity Labeling of EF-2—The reaction was performed in Eppendorf tubes containing 50 μl of 20 μM EF-2 in 30 mM Tris-HCl, pH 7.5, 10 mM KCl, 200 μM EDTA, 20% (v/v) glycerol, 10 mM β-mercaptoethanol and the appropriate amount of [14C]GM258383. After preincubating for 15 min at 25 °C in the dark, samples were placed on ice and irradiated at 254 nm with an UV lamp (2000 microwatts/cm²). Further sample processing was dependent on the aim of the experiment. For quantification purposes, samples were denatured by adding 150 μl of 8 M guanidine chloride followed by 5 min of heating at 80 °C. Then, free drug was removed by gel filtration through Sephadex G-25, and the extension of covalent labeling was determined by liquid scintillation counting. For other purposes samples were processed as opportunistically described.

Identification of the Photolabeled Residue—Photolabeling of EF-2 was performed as described above using 100 μM [14C]GM258383 and 10-min UV-irradiation. Gel filtration on Sephadex G-25 was then used to remove drug excess and to exchange buffer to 100 mM ammonium acetate, pH 5.0. Further reduction and alkylation with 4-vinylpyridine, trypsin digestion, and reverse-phase HPLC were performed according to established methods (23). Amino acid sequences were determined with an Applied Biosystems pulse-liquid sequencer model 477A connected on-line to a reverse-phase HPLC unit for identification of the stepwise released phenylthiohydantoin-amino acids. Mass determinations were performed with a Bruker Biflex III MALDI time of flight mass spectrometer. These analysis were done by Eurosequence b.v. (Groningen, The Netherlands).

RESULTS AND DISCUSSION

GM258383, the photoactivatable aryl azide sordarin derivative used in the present work (see structure in Fig. 1), inhibits protein synthesis in C. albicans cell-free systems (IC50 = 2 μM, determined as in Ref. 20). This biological activity makes GM258383 appropriate for studies intended to identify sordarin binding site. Ideally, this photoprobe was intended to label the ribosome-EF-2 complex, because this is considered the functional target of sordarin. However, the spectral properties of GM258383 (maximum absorption at 260 nm and no absorption at wavelengths longer than 300 nm) are almost identical to those of ribosomes; consequently, photoactivation of the probe is quenched by the inner filter effect of ribosomes. Indeed, all efforts to photolabel mixtures of ribosomes and EF-2 were unsuccessful (data not shown). Therefore, labeling experiments were carried out with EF-2 alone.

To characterize the binding of sordarin to its target we have performed equilibrium dialysis experiments with [3H]sordarin and either EF-2 alone or equimolar mixtures of EF-2 and ribosomes in the presence of a non-hydrolyzable GTP analog, i.e. Gpp(NH)p (Fig. 2). Sordarin binding to EF-2 alone showed saturation, the Scatchard plot corresponding to that of one single class of binding sites (Fig. 2A). Fitting the experimental points to a single hyperbola yielded Kd = 1.26 μM. This indicates that sordarin binds to EF-2 in a specific manner to a
defined binding site within the EF-2 molecule and hence supports performing photoaffinity labeling with EF-2 alone. This affinity, similar to that described for rat liver EF-2 and its natural substrate GTP (\(K_d = 3.0 \mu M\)) (24), seems to be high enough to reduce the risk of nonspecific labeling (25, 26). On the other hand, the presence of ribosomes unveils two classes of binding sites with higher affinity, as deduced from the Scatchard plot (Fig. 2B). \(K_d\) values for these two sites (calculated from data fitting to a double hyperbola) were 0.7 and 41.5 nM. These values are far apart with respect to the value with EF-2 alone and might be indicative of the presence of two populations of ribosomes (pre- and post-translocated) in agreement with our previous observations (4). The affinity increase may result from conformational changes within EF-2 upon interaction with the ribosome rather than from the creation of a new site in EF-2, because the latter seems unlikely in view of the already notable affinity shown by EF-2 alone. The possibility of a combined binding site in the interface between ribosome and EF-2 cannot be ruled out either. Interestingly, it has been recently shown that the sordarin derivative GM193663 increases the reactivity of 26 S rRNA in Saccharomyces cerevisiae ribosomes, most notably affecting to the sarcin-ricin loop, which becomes more exposed (27).

The use of aryl azides (such as GM258383) in photoaffinity labeling is widespread. Nevertheless, the long lifetime of the reactive intermediate has been deemed a serious caveat that may lead to nonspecific labeling (28). Fig. 3A shows EF-2 photolabeling with \([^{14}C]GM258383\) at different irradiation times. Labeling followed pseudo-first order kinetics with \(k_{obs}\) of 0.215 min\(^{-1}\) and was quantitatively completed after 10 min. When EF-2 was irradiated in the presence of increasing concentrations of \([^{14}C]GM258383\), a hyperbolic saturation curve was obtained (Fig. 3B), with maximum incorporation at 0.39 mol/mol EF-2. The most convincing result to support the specificity of photolabeling with \([^{14}C]GM258383\) is presented in Fig. 3C, where it is demonstrated that sordarin derivative GM193663 prevented photolabeling in a dose-dependent manner, so that incorporation of the photoprobe was completely precluded in the presence of high concentrations of GM193663 (Fig. 4, lane 3). On the other hand, when the reaction mixture was not irradiated at 254 nm no labeling was detected by SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 4, lane 4). In all, it is proved that GM258383 is covalently bound to a EF-2 sordarin binding site as a result of a photoactivatable process.

To identify the EF-2 residue modified by \([^{14}C]GM258383\),
photolabeled EF-2 was digested with trypsin and the resulting peptide mixture was resolved by reverse-phase HPLC (Fig. 5). Five fractions (named “A” to “E”) showed significant radioactive levels, which accounted for more than 60% of the total. Besides, there is a spurious trail of radioactivity at the end of the chromatogram, which is not associated with UV peaks at 214, 254, 280, or 297 nm; hence, it might be attributed to traces of free decomposed radioligand. Fraction A was further purified by HPLC (Fig. 6) and yielded two radioactive peaks, “A1” and “A2.” Sequence analysis of these two peaks, by Edman degradation, rendered uFANxYSK (peak A1; where “u” denotes no unambiguous assignment and “x” denotes no detection) and FANxYSKK (peak A2). MALDI-MS of peak A1 rendered a signal at \( m/z = 1437 \) (MH\(^+\)) attributable to the mass of the peptide QFANKYSK (986 Da; where “q” is pyroglutamic acid) plus the mass of the photoprobe (450 Da). Transformation of Gln into Glu and subsequent lactam formation blocked the N terminus of the peptide, thus preventing Edman degradation. Finally, when the rest of the radioactive peaks were analyzed in the same form, either no radioactivity was detected in the re-chromatography, or no sequence nor MS results were obtained. This suggests that these peaks might correspond to modified peptides present in scarce amounts.

The modified Lys\(^{228}\) is located in the G-domain (also called domain I) of EF-2, more precisely at the beginning of the insert called G\(^{11033}\)-subdomain. The analog insert in bacterial EF-G (called the G\(^{11032}\)-subdomain) is located before the G5 motif, between helix D5 and strand 6G (see Ref.9). However, in eukaryotic EF-2, the insert is longer and placed after the G5 motif. Because this G\(^{11033}\)-subdomain is involved in sordarin binding, such differences between G\(^{11033}\)- and G\(^{11032}\)-subdomains can play a role in determining the innocuousness of sordarins on bacterial protein synthesis.

Fig. 9 shows the alignment of the G\(^{11033}\)-subdomains from all EF-2 sequences presently available. It is noteworthy that such a subdomain is longer in EF-2 from higher animals than in the rest of eukaryotic species due to a 13-residue fragment located at the
beginning of the subdomain. More remarkable is the existence of two putative Walker motifs (29), with Walker-A being present only in this group of species. Recently, Gonzalo et al. (30) identified in rat liver EF-2 a second nucleotide binding site specific for ATP, which may probably involve these additional Walker motifs. Although the underlying physiological function is not known, ATP hydrolysis (35). Therefore, these mutations in EF-2 may help to overcome the effect of the drug by affecting EF-2 function rather than sordarin binding to EF-2 alone, thus precluding the transition to a high affinity sordarin binding complex upon interaction with the ribosome. On the other hand, there are five more sordarin-resistant mutations out of domain III, two of them located near the photolabeled peptide (6).

In summary, the results presented in this paper may contribute to the sordarin mode of action. In any case, studies designed to afford a more detailed definition of the molecular effects of this drug at ribosomal level are still needed.

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