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HEPATIC CYTOCHROME P450 UBIQUITINATION: CONFORMATIONAL PHOSPHODEGRONS FOR E2/E3 RECOGNITION?

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Abstract

Hepatic endoplasmic reticulum (ER) integral cytochromes P450 (P450s) are monooxygenases engaged in the biotransformation and elimination of endo- as well as xenobiotics. Of the human liver P450s, CYP3A4 is the major and most dominant catalyst, responsible for the biotransformation of over 50% of clinically prescribed drugs. CYP2E1 metabolizes smaller molecular weight compounds (EtOH), carcinogens, environmental toxins and endobiotics, and is justly implicated in various toxigenic/pathogenic mechanisms of human disease. Both P450s are notorious for their potential to generate pathogenic reactive oxygen species (ROS) during futile oxidative cycling and/or oxidative uncoupling. Such ROS not only oxidatively damage the P450 catalytic cage, but on their escape into the cytosol, also the P450 outer surface and any surrounding cell organelles. Given their ER-monotopic topology coupled with this high potential to acquire oxidative lesions in their cytosolic (C) domain, not surprisingly these P450 proteins exhibit shorter lifespans and are excellent prototype substrates of ER-associated degradation ("ERAD-C") pathway. Indeed, we have shown that both CYP3A4 and CYP2E1 incur ERAD-C, during which they are first phosphorylated by protein kinases A and C, which greatly enhance/ accelerate their ubiquitination by UBC7/gp78 and UbcH5a/CHIP/Hsp70/Hsp40 E2/E3 ubiquitin ligase complexes. Such P450 phosphorylation occurs on Ser/Thr residues within linear sequences as well as spatially clustered acidic (Asp/Glu) residues. We propose that such S/T phosphorylation within these clusters creates a negatively charged patch i.e. conformational phosphodegrons, for interaction with positively charged E2/E3 domains. Such P450 S/T phosphorylation we posit serves as a switch to turn on its ubiquitination and ERAD-C.

INTRODUCTION

Cytochromes P450 (P450s; CYPs) are hemoprotein enzymes ubiquitously found in nature (i.e. bacteria, plants, yeast, fish, avians and mammals). They function largely but not exclusively as mixed function oxidases, engaged both in synthetic and degradative functions of key importance to the cellular integrity, physiology and defense (1, 2). Although P450s

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are present in various organs and intracellular loci, those discussed in this article belong to the P450 family residing in the endoplasmic reticulum (ER) of the mammalian liver, wherein they function in concert with their redox partners cytochrome P450 reductase (CPR) and cytochrome b_5 (b_5) in the oxidative metabolism and elimination of numerous endobiotics (arachidonic acid, retinoic acid, steroids, vitamin D) as well as xenobiotics (pharmacological and recreational drugs, carcinogens, toxins and other foreign substances of dietary or environmental origin) (1, 2). These substrates can modulate hepatic P450 content, diversity and/or function through induction via either increased protein synthesis/expression or protein stabilization i.e. half-life prolongation (1–5). By contrast, "suicide" substrate/inactivators accelerate the proteolytic degradation of certain P450s and dramatically shorten their half-lives (6–13). Notably, such substrate-mediated P450 induction, stabilization or enhanced turnover by controlling the hepatic content of specific P450s, not only influence the effectiveness of drugs and/or the duration of their pharmacological response, but also the severity and the time course of many pharmacokinetic/pharmacodynamic drug-drug interactions. Because of these clinically relevant issues (14–17), prescreening for potential hepatic P450 effects is a prime and required therapeutic consideration in the design, development and preclinical evaluation of any potential drug candidate.

Hepatic P450 degradation: Cellular pathways

The hepatic ER-membrane anchored P450s (M.W. $\approx$ 50 kDa) are excellent examples of Type I monotopic proteins with their first N-terminal $\approx$ 30-33 amino acid residues incorporated in the ER-membrane, and the bulk of their globular structure enveloping the catalytic core, exposed to the cytosol (1, 18). Although all hepatic P450s are integral ER-proteins, their individual lifespans with half-lives ($t_{1/2}$) ranging from 7-38 h are highly variable (18, 19). The relatively shorter lifespans of some P450s are thought to stem from the leakage of powerful reactive oxygen species (ROS), engendered during the course of their futile catalytic cycling in the absence of a substrate, or even upon oxidative uncoupling in the presence of a weakly/poorly accepted substrate. Such ROS can oxidize active site residues and/or P450 protein surface residues, thus triggering the subsequent triage of the irreversibly damaged autooxidized protein. Accordingly, this particular ER-topology of P450s coupled with their propensity for active site structural lesions within their cytosolic (**C**) domain upon either oxidative injury during catalysis or mechanism-based inactivation by "suicide" substrates, would *a priori* qualify them as excellent candidates for ER-associated degradation (ERAD) via the ubiquitin (Ub)-dependent proteasomal system (UPS), specifically the "ERAD-C" pathway (20–24). Intriguingly however, although all hepatic ER-anchored P450s exhibit this very Type I topology, not all of these liver proteins are ERAD-C substrates. Some such as the rat CYP2B1 and CYP2C11 have longer $t_{1/2}$s ($\approx$ 20-37 h) and incur autophagic-lysosomal degradation (ALD) (18, 25–28). The structural determinants if any, of this divergent proteolytic targeting remain to be identified. Plausibly, the protein stability of these particular ALD-prone P450s is due to their inherently lower propensity to generate ROS during catalysis and thus a lesser susceptibility to autooxidative damage. These features in turn enable their longer ER-residence and associated ER-membrane turnover via ALD.

Our most recent studies have specifically focused on the proteolytic turnover of human liver CYP3A4 and CYP2E1 and their corresponding rabbit/rat/mouse liver CYP3A and CYP2E1 orthologs (29–36). CYP3A4 is the major human liver and intestinal drug metabolizing P450 enzyme accounting for $\approx$ 30% of the hepatic P450 content and engaged in the metabolism of over 50% of clinically relevant drugs and other xenobiotics (1, 2). Our findings in various *in vivo* and *in vitro* reconstituted eukaryotic systems have documented that both native and structurally/functionally inactivated CYPs 3A incur Ub-dependent 26S proteasomal degradation (UPD; 29–36), in a typical ERAD-C process (21–23). Substrates such as

troleandomycin, ketoconazole, and clotrimazole that bind tightly to CYP3A prosthetic heme and/or active site tend to stabilize the enzyme protein, reduce its oxidative turnover and extend its half-life (3, 31, 37). Furthermore, conditional deletion of the liver CPR gene in mice also elevates their constitutive hepatic CYP3A content (38, 39), plausibly due to reduced futile oxidative cycling and consequent protein stabilization. By contrast, mechanism-based inactivators such as the grapefruit juice furanocoumarins and DDEP (3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine; 7, 8), which structurally and/or functionally inactivate CYPs 3A, accelerate their proteolytic degradation and disposal, depleting hepatic CYP3A content.

Human liver CYP2E1 is a P450 enzyme responsible for the biotransformation of clinically relevant low molecular weight drugs (acetaminophen, halothane, sevoflurane, chlorzoxazone), ethanol (EtOH), and other xenobiotics, carcinogens (nitrosamines) and endogenous ketones (acetone) and fatty acids (arachidonic acid) (1, 40, 41). Its notorious capacity for bioactivation of xenobiotics into toxic/reactive intermediates, coupled with its high propensity for ROS generation have convincingly implicated CYP2E1 in pathogenic mechanisms of toxic liver damage, alcoholic liver disease, diabetes, and obesity (40–42). Its content, normally $\approx$ 7% of the total hepatic P450 content, is also increased in these conditions, thereby predisposing and abetting vicious pathogenic cycles of liver injury (1, 40, 41). Thus tight regulation of CYP2E1 content may be viewed as clinically desirable. Such regulation involves CYP2E1 protein synthesis and degradation via both ERAD/UPD and ALD (9–11, 26, 35). Rat liver CYP2E1 undergoes biphasic degradation exhibiting a rapid phase ($t_{1/2}$, 7 h) and a slow phase ($t_{1/2}$, 37 h) that reflect its degradation via ERAD/ UPD and ALD, respectively (4, 5, 18). The rapid phase apparently stems from structural damage inflicted by ROS generated during its futile oxidative cycling either in the absence or on withdrawal of an appropriate substrate (i.e. EtOH).

Accordingly, substrate binding at its active site stabilizes the CYP2E1 protein and prolongs its half-life (4, 5, 35, 42), diverting it into the slower ALD pathway. Furthermore, functional inactivation of its catalytic redox partner CPR abolishes all electron-flux, thereby decreasing futile oxidative cycling, stabilizing CYP2E1 and prolonging its half-life (43, 44). Nevertheless, we found that heterologously expressed human CYP2E1 in spite of its sluggish catalytic turnover in *S. cerevisiae* due to insufficient cellular CPR content (35) still turns over via both ERAD and ALD pathways. This infers the additional existence of ERAD/ALD determinants or "degrons" (35).

Our molecular dissection of hepatic CYP3A and CYP2E1 ERAD-C process revealed that it involves posttranslational phosphorylation of both P450s by cytosolic kinases (32–36), ubiquitination by the ER-integral gp78/AMFR ("glycoprotein" 78/autocrine motility factor receptor) as well as the cytosolic CHIP (C-terminus of Hsp70-interacting protein) E3 Ub-ligases and their respective cognate E2 Ub-conjugating enzymes UBC7/Ube2g2, and UbcH5a respectively (34–36; 45, 46), and subsequent degradation by the 26S proteasome (8, 18, 19, 34–36; Fig. 1). The monotopic ER-topology of the P450 proteins with the bulk of their structure exposed to the cytosol should render them quite accessible to the 26S proteasome, which is often closely associated with the ER-membrane (47, 48). Nonetheless, we found that their ER-membrane extraction into the cytosol by the p97 AAA ATPase-Npl4-Ufd1 chaperone complex is required for P450 proteasomal processing (31, 49), just as in the retrotranslocation of polytopic transmembrane and/or luminal ER-proteins (50–53). Accordingly, hepatic p97 knockdown (>90%) almost completely abrogated CYP3A extraction and subsequent proteasomal degradation, resulting in the accumulation of both unmodified, functionally active parent and ubiquitinated CYP3A species integrated in the ER-membrane (49).

ERAD, an important determinant of hepatic P450 content and function

Until recently, the prevailing notion was that ERAD/UPD of P450s served essentially to dispose off inactivated, and/or structurally damaged P450s past their useful prime, thereby clearing undesirable, unwanted cellular/ER-garbage (18, *and references therein*). Indeed, ERAD/UPD plays a key role in the clinically relevant polymorphic expression of human liver P450s through removal of various defective, misfolded and/or proteolytically susceptible P450 variants (17, *and references therein*). Such a funerary function of P450 ERAD is vital for quality control and to ward off proteotoxic stress due to the accumulation of abnormal/aberrant and/or structurally damaged proteins. However, our recent findings reveal two even more important features of P450 ERAD/UPD, both of which contribute towards the regulation of hepatic microsomal P450 content and thus its function, and as such are clinically and pathologically relevant: The first is that not all *de novo* synthesized P450 proteins are incorporated into the ER-membrane. In fact a significant fraction of newly synthesized ^{35}S-labeled CYP3A immediately following ^{35}S-pulse-chase of cultured rat hepatocytes are ubiquitinated as documented by high molecular mass (HMM) ^{35}S-CYP3A species (31). This suggests that P450 UPD controls the amount of the newly synthesized/ folded protein that is incorporated into the ER-membrane at any given time. The second is our finding that lentiviral shRNA interference (shRNAi) directed against either gp78 or CHIP E3 Ub-ligase markedly increases functionally active hepatic ER-anchored CYP3A content (46; *see below*). This indicates that each of these E3s in addition to targeting fatally damaged P450s, also targets either native P450s or an intermediate species, and thus regulates functional hepatic P450 content. These findings provide compelling direct evidence for ERAD/UPD as an important physiological regulator of hepatic P450 content and function, and not just a P450 mortuary operator. Moreover, such a physiological regulation also implies that any impairment of P450 ERAD and consequent elevation of functional hepatic P450 content would as discussed earlier not only be relevant to clinical therapeutics, but also have pathological implications due to the propensity of some P450s such as CYP3A4 and CYP2E1 to generate pathogenic ROS.

Hepatic P450 ubiquitination: Cellular roles of gp78 and CHIP E3 Ub-ligases

gp78 E3 Ub-ligase is a polytopic membrane-anchored protein present largely in the ER, but apparently also at the cell surface (53–59). gp78 is N-terminally ER-anchored [amino acids (aa) 1-308] with its intrinsic RING-finger Ub-ligase (aa 341-378), Cue1-like (aa 456-497), UBC7/Ube2g2-binding (G2BR, aa 579-600), substrate recognition (aa?) and putative p97-binding regions (aa 626-643) all situated within its cytosolic C-terminal 309-643 residue region, the functionally active E3 domain (53, 56, 57, 59). Its primary catalytic role is to recruit a prospective substrate and bring it in close proximity to its bound cognate UBC7 E2 thereby promoting the *"en bloc"* transfer of an UBC7-elaborated polyUb-chain onto one or more substrate Lys (K)-residues (53, 57, 60). Its ER-topology, UBC7-dependence and functional p97 association, are all consistent with the known requirements for CYP3A ERAD (Fig. 1). Indeed, our shRNAi analyses of gp78 in cultured rat hepatocytes not only have established its physiological role in CYP3A ERAD, but also have revealed that gp78 knockdown of ≈ 80% results in CYP3A stabilization with increased ER-levels of a functionally active enzyme (46). This occurs in spite of the presence of CHIP and any other E3s putatively involved in ERAD, thus underscoring that the gp78 function is essential rather than redundant in CYP3A ERAD.

Accordingly *in vitro* incubation of structurally inactivated human liver CYP3A4 in an UBC7/ gp78-ubiquitination system functionally reconstituted with purified Ub-activating E1 enzyme, Ub and ATP, followed by tryptic/lysyl endopeptidase C (Lys-C) digestion of the incubated CYP3A4 protein and subsequent HPLC-MS/MS analysis of this protein digest to

monitor for Ub-derived GG- or LRGG-remnant modified CYP3A4 peptides, led to our identification of CYP3A4 K115, K168, K282 and K492 as gp78-ubiquitination sites (36). Corresponding analyses of CYP2E1 in a similarly reconstituted UBC7/gp78-ubiquitination system led to our identification of K84, K87, K275, K410, K420, K422, K428, K434, and K461 of human liver CYP2E1, as well as K275 and K434 of rabbit liver CYP2E1 as the specific gp78-ubiquitination sites (35).

CHIP on the other hand, is an UbcH5a-dependent U-box E3 Ub-ligase that functions cooperatively with Hsp70/Hsp40 as a cochaperone in UPD substrate-recruitment (12, 61–65). CHIP is a 303-residue long 38.4 kDa cytosolic protein containing three tandem tetratricopeptide repeats (TPR) in its N-terminal domain (aa 1-197) for Hsc/Hsp70- and Hsp90-binding, and a very highly (>87% from flies to human) evolutionarily conserved C-terminal RING-finger like U-box (aa 210-303). A role for CHIP in CYP3A4 ERAD was not predicted *a priori* from our initial *S. cerevisiae* degradation analyses because yeast lacks CHIP (21). However, our preliminary findings of CYP3A4 stabilization in a temperature sensitive Hsp70-defective yeast mutant (*ssa1-45*; kindly provided by Prof. J. Brodsky), coupled with CHIP's ability to ubiquitinate not only CYP3A4 (36, 45, 46) but also CYP2E1 (12, 35) in *in vitro* functionally reconstituted ubiquitination systems (Fig. 2), suggested its involvement in P450 ERAD *in vivo* Fig. 1). Indeed, our findings that lentiviral shRNAi-elicited hepatic CHIP knockdown of $\approx$ 80% also led to a corresponding $\approx$ 2.5-fold stabilization of functionally active CYP3A (46) and CYP2E1 content (*S. M. Kim and M. A. Correia, preliminary findings*), verifies a physiological role of CHIP in P450 ERAD. Once again its function, as that of gp78, was also not redundant and/or compensated by any of the numerous cellular E3s (including hepatic gp78), functional in ERAD.

Intriguingly however, whereas shRNAi-elicited gp78-knockdown in cultured rat hepatocytes considerably reduced CYP3A export out of the ER into the cytosol, and thus its subsequent 26S proteasomal degradation, significant CYP3A ubiquitination (detected as HMM ^{35}S-species) still persisted both in the ER and cytosol (46). This indicated that gp78 knockdown reduced but did not abolish CYP3A ubiquitination and ER to cytosol export, altogether (46). By contrast, parallel shRNA-mediated CHIP knockdown completely abrogated both CYP3A ubiquitination and its export into the cytosol (46). These findings reveal that "*in vivo*" CHIP may function quite early to prime CYP3A ubiquitination in the ER, and that after such initial CHIP-mediated CYP3A priming, gp78 may serve as an E4 to extend the already initiated Ub-chains (56, 57). However, the finding that gp78 can *per se* effectively ubiquitinate P450s (and its other substrates) in *in vitro* reconstituted systems suggests that it can function both as an E3 and an E4 in P450 ERAD/UPD (34–36, 45, 46).

LC-MS/MS analyses of each P450 incubated with CHIP, its cognate E2 UbcH5a and Hsp70/Hsp40 in an *in vitro* functionally reconstituted ubiquitination system very similar to that described above, indicated that CHIP ubiquitinated human liver CYP3A4 at residues K127, K168, K173, K466, K487 and K492, and human liver CYP2E1 at K87, K251/255, K275, K410, K420, K428 and K434 (35, 36). K251, K255 and K275 of rabbit liver CYP2E1 were similarly ubiquitinated (35). Surprisingly, this list of ubiquitinated CYP2E1 K-residues did not include K317 and K324 previously identified as putative CYP2E1 ubiquitination sites on the basis of homology modeling prediction and the ability of an antibody raised against CYP2E1 310-340 peptide to block *in vitro* ubiquitination of this enzyme in a rabbit reticulocyte lysate system (66). More recently, similar multisite K-ubiquitination by CHIP of the P450-like neuronal nitric oxide synthase (nNOS) has also been reported (67). Strikingly, key K-residues were identified in the nNOS calmodulin-binding domain, which are essential for its UPD (67).

Additional salient features of P450 ubiquitination by these two E2/E3 systems include: First, only a few of the total 38 K-residues in the CYP3A4 and 29 K-residues in the CYP2E1 primary structure, were detected as targets upon *in vitro* ubiquitination (35, 36), even though these P450 proteins were purified i.e. extracted, and thus unencumbered by the ER-membrane. Second, although these two E2/E3 systems were found to ubiquitinate distinct CYP3A4 K-residues (i.e. K115 and K282 by gp78, vs K127, K173, K466, and K487 by CHIP), they also targeted the very same residues (K168 and K492) (36). This was also true of the corresponding ubiquitination of CYP2E1 by gp78- and CHIP systems (35). These findings thus reveal that at the least *in vitro*, the P450 protein interfaces were not mutually exclusive to either E2/E3 complex, with K-residues on both the proximal and distal P450 surface susceptible to ubiquitination. Whether this would hold true *in vivo* remains to be determined. Admittedly, peptide antibody mapping studies coupled with structural analyses suggest (68–71) that *in vivo*, in addition to the ER-membrane anchor, significant outer surfaces of the hepatic P450s (for instance, the G'-helix and the loops following helix A'' and between the G' and G helices of CYP3A4) being hydrophobic are most likely embedded in the ER-lipid bilayer (72–74). *In vivo*, this would preclude direct protein-protein interactions of these P450 surfaces not only with their redox partners, but also with the E2/E3 ligases. Surprisingly, we found that none of the K-residues in these P450 regions were in fact ubiquitinated even in the purified, membrane-free proteins. Thus, as in the case of inositol 1,4,5-trisphosphate (IP_3)-receptor ubiquitination (75), the P450 K-residues that were targeted are all situated on cytosol-exposed surface loops accessible both to the cytosolic and ER ubiquitination machinery (35, 36). However, it is unclear which of the P450 K-residues targeted by gp78 and/or CHIP were polyubiquitinated or mono-ubiquitinated, and if there is any one critical K-residue whose ubiquitination is the *sine qua non* for P450 degradation, as recently reported for nNOS (67). When the structural composition of the polyUb-chains modifying each P450 were specifically analyzed by LC-MS/MS, it was found that the gp78 system strictly elaborated K48 Ub-linkages that are generally associated with 26S proteasomal targeting (35, 36). The CHIP system on the other hand, generated K48, K63 and K11 Ub-linkages, consistent with its functional involvement in both UPD and ALD pathways (35, 36).

Together these findings reveal that both hepatic gp78 and CHIP have individually important albeit complementary roles in CYP3A and CYP2E1 ERAD. They also provide proof of principle that functional disruption of each of these E3 enzymes is a means to specifically down regulate P450 ERAD. Surprisingly however, CYP3A stabilization following shRNAi of either gp78 or CHIP resulted in increased ER-content of functionally active CYP3A, thereby challenging the prevailing notion that E3-recognition and subsequent ERAD of P450 proteins requires *ab initio* their structural and/or functional inactivation. The critical determinant of the precise timing during the cellular lifespan of a functional P450 when it is first committed to triage remains however to be identified. It is conceivable that: (i) in addition to targeting an inactive, structurally damaged P450 species, each E3 Ub-ligase also directly recruits a native, structurally and functionally intact P450 species for ERAD, as proposed for other "wild type" proteins (21); or (ii) when its ERAD is disrupted, the structurally/ functionally intact CYP3A protein accumulates in the ER largely because its subsequent progress to the critical cellular intermediate species that is specifically recognized by either gp78 or CHIP E3 Ub-ligase is stalled. Our findings indicate that a multisite phosphorylated P450 protein may be a fitting candidate for this intermediate species (33–36).

Multisite protein phosphorylation for P450 recognition by gp78 and CHIP E3 Ub-ligase as an ERAD substrate

Since its first discovery in 1983, P450 phosphorylation has been ascribed various roles (76–80) including targeting the protein to degradation by a microsomal Ser-protease (81), cytosolic ATP-dependent protease (82) or as suggested by us, UPD (32–36). However, the precise role of protein phosphorylation in P450 ERAD/UPD remained a mystery. Our recent findings that S478 phosphorylation of CYP3A4 both accelerates and enhances its gp78-mediated ubiquitination, events mimicked by its phosphomimetic S478D mutant as well as CYP3A5 (its natural phosphomimetic D478-containing human liver ortholog), but not its phosphorylation-impaired S478A mutant, reveal a critical role of S478-phosphorylation in CYP3A4 recognition by gp78 (Fig. 3; 34, 36).

We have previously documented that protein kinase (PK) A phosphorylates CYP3A4 S478 *in vitro* (34, 36). Although this PKA-mediated S478-phosphorylation may not be physiologically relevant, our proteomic findings in cultured rat hepatocytes indicated that S479, the corresponding rat liver CYP3A23 residue, is also phosphorylated "*in vivo*" by a hepatic kinase (36). We have also previously documented that the phosphorylation of CYP3A4 S420 and T264 by PKC synergizes that of S478 in enhancing gp78-mediated CYP3A4 ubiquitination and ERAD (34, 36). However, despite their relevance in CYP3A4 ubiquitination, S478, S420 and T264 are just 3 of the 15 CYP3A4 residues actually phosphorylated by PKA/PKC *in vitro*. It is currently unknown whether the phosphorylation of any of the other 12 residues [T92, S100, T103, S116, S119, S131, T136, S139, S259, T284, S398 identified by us (36), and S134 by us and others as well (36, 83)] is also relevant to gp78-mediated CYP3A4 ubiquitination or whether the phosphorylation of S478/S420/T264 suffices.

Our proteomic studies (35) have similarly identified 16 new sites (S56, T58, T69, S74, T121, T131, T132, S145, S247, S256, T373, T376, T387, S424, S431, T432) upon *in vitro* PKA/PKC-catalyzed CYP2E1 phosphorylation in addition to the previously identified S129 (76, 83–85). Similarly, CYP2E1 phosphorylation also significantly enhanced its gp78-mediated ubiquitination (35; Fig. 2). The precise phosphorylation sites (analogous to CYP3A4S478), critical for this enhanced CYP2E1 ubiquitination are presently unknown. However, of these CYP2E1 sites, S129, in spite of its relatively high extent of phosphorylation (98.2% by PKA and 23.5% by PKC; 35), may be securely excluded as relevant, as its mutation had little influence on CYP2E1 protein stability (85).

Together, our findings that gp78-mediated P450 ubiquitination is enhanced/accelerated not only by the phosphorylation of both P450s (Fig. 2), but also by the phosphomimetic CYP3A4S478D mutant, whereas it is abrogated by CYP3A4S478A mutation (34, 36), argue for the existence of specific gp78 domains for the recognition of phosphorylated or phosphomimetic P450 residues. The relevant gp78 domains for such P450 phosphodegron recognition presently remain to be identified. Nevertheless, we believe that our finding that P450 protein phosphorylation both accelerates and enhances its gp78-mediated ubiquitination may provide some crucial insight into the as yet obscure mechanisms of gp78-mediated targeting of its other ERAD substrates for ubiquitination.

We have also documented that CHIP-mediated ubiquitination of both P450s is also enhanced upon inclusion of PKA/PKC in the incubation (35, 36; Fig. 2). This suggests that protein phosphorylation may also promote P450 interactions with CHIP itself, UbcH5a (its cognate E2), and/or Hsp70, its cochaperone partner. However, neither CYP3A4S478 alone or in combination with T264 and S420 was found to be involved in this enhancement as judged by the failure of the corresponding Ala-mutants to appreciably affect CHIP-mediated

CYP3A4 ubiquitination (Fig. 3; 36). Thus it remains to be determined which of the other 12 CYP3A4 phosphorylated residues if any, are involved in promoting CYP3A4 interactions with CHIP and/or Hsp70. The reports that CHIP recognition of the human androgen receptor as well as hyperphosphorylated Tau (the pathologic component in Alzheimer's disease neurofibrillary tangles) occurs in a highly specific, multisite phosphorylation- and sequence-dependent context i.e. "distributed phosphodegrons" (86, 87), supports this possibility.

Phosphorylation could in principle synergistically enhance CHIP-mediated CYP3A4 ubiquitination by also enhancing CYP3A4's interactions with Hsp70 and/or UbcH5a. Indeed, we have recently found that the initial interactions between Hsp70 and CYP3A4WT are enhanced as expected not only by its inactivation (which exposes hydrophobic core regions), but also by CYP3A4 phosphorylation. However, Hsp70-interactions with structurally inactivated CYP3A4WT, and its T264A/S420A/S478A, S478D or S478A mutant (each monitored in parallel following incubation with PKA/PKC, reversible cross-linking with paraformaldehyde, CYP3A4 immunoprecipitation, SDS-PAGE and Hsp70 immunoblotting) were found to be comparable. This suggests that most likely, phosphorylated residues other than the above three may be involved in its Hsp70-interactions (*YQ. Wang and M. A. Correia, preliminary findings*). Indeed, this possibility is consistent with the failure of the T264A/S420A/S478A mutant to appreciably affect CHIP-mediated CYP3A4 ubiquitination (Fig. 3; 36).

P450 conformational phosphodegrons for molecular recognition by the E2/E3 complexes?

Inspection of some of the available CYP3A4 and CYP2E1 crystal structures (40, 72–74) reveals that not only the ubiquitinated K-residues, but also the Ser and Thr (S/T)-residues phosphorylated in each protein reside on surface loops within a cluster of acidic [Asp (D), Glu (E)] residues (Fig. 4). Thus by imparting additional negative charges, S/T-phosphorylation would further augment the negatively charged character of this D-E-S/T cluster. Accordingly we hypothesize that (i) such acidic P450 surface patches may indeed be important for its ERAD substrate recognition by corresponding basic residues/domains in the E3 Ub-ligases, their cognate E2 Ubcs, and/or chaperone partners; and (ii) that S/T-phosphorylation by filling in the missing gaps in the negatively charged clusters would control the timing of P450 recognition by the E2/E3 complexes and thus function as a "switch" to turn on P450 ERAD/UPD.

We find it noteworthy, that in addition to phosphorylatable residues, two consecutive acidic residues (E, D) at -2 and -1 position of an ubiquitinatable K-residue (position 0) are found in yeast integral membrane proteins (88). Similarly, K-residues of HMGCoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase in KEEE or KNEEEE motifs were found preferentially ubiquitinated by gp78 (89). Furthermore, a positively charged residue in the immediate vicinity of the E2-catalytic Cys bearing the dischargeable activated Ub-thioester species, serves as a "hook" for an acidic residue in the proteins ubiquitinated *solely* by Ubc4 or Ubc5 (88). Intriguingly, gp78-ubiquitinated CYP3A4 K115 lies within a spatially associated surface cluster of acidic/phosphorylatable residues (Fig. 4). Similarly, CYP3A4 K282 residing in the "disordered" H-helix region in the crystal structure (71–73) is flanked by S281 and E283-T284-E285-S286, in the close proximity to another negatively charged E258-S259-E262-D263-T264 surface cluster (Fig. 4). Interestingly, CYP3A4 S259, T264 and T284 within these clusters are very highly phosphorylated by PKC (36). Furthermore, our preliminary LC-MS/MS analyses of gp78-cross (**X**)-linked CYP3A4S478D with the "0-length" X-linker EDC (1–ethyl-3-3-[3-dimethyl-aminopropyl]carbodiimide HCl; 90) have led us to consistently identify CYP3A4 peptide regions R106-K127 and V269-K282 as gp78-interacting domains (*YQ. Wang, S. Guan and M. A. Correia, unpublished*

observations). These regions harbor CYP3A4 ubiquitinated residues K115 and K282, thereby verifying their direct interactions with gp78.

Interestingly as well, the CYP3A4 R106-K127 region also harbors K127, a residue ubiquitinated by the CHIP system (36). Similar analyses of other CHIP-ubiquitinated CYP3A4 K168, K487 and K492 reveal that they all also reside within surface clusters comprised of E163-E165-D174-D214-D217-E303-E308 and E470-S478-E486-E494-D497 (Fig. 4). The latter cluster lies in the CYP3A4 C-terminal loop, a region of "*profound structural plasticity*" (74). As underscored above, phosphorylation of S478 within this cluster was found to be critical for CYP3A4-gp78 interaction (Fig. 3), and K492 in this C-terminal loop was indeed targeted for gp78 ubiquitination (36). However, even though K487 and K492 within this C-terminal loop were also ubiquitinated by CHIP (36), such CHIP-mediated CYP3A4-ubiquitination was not at all dependent on S478 phosphorylation (Fig. 3). Thus, the roles of particular surface E-D-S/T clusters that facilitate P450 interactions with each E2/E3 complex may vary, with some clusters on one P450 interface being more dominant in gp78 interactions, whereas others on a different P450 interface facilitating CHIP interactions.

Scrutiny of the CYP2E1 structure (40) also reveals that every CYP2E1 K-residue ubiquitinated by either gp78 or CHIP similarly lies within such acidic D-E-S/T surface clusters (Fig. 4; 35). Although some of these P450 clusters indeed occur in a linear sequence conforming to a canonical "phosphodegron", others are assembled together by the P450 tertiary structural fold. We therefore propose that these contiguous, negatively charged and spatially associated clusters serve as "conformational phosphodegrons" and are important for P450 recruitment by each E2/E3 ubiquitination system. Thus we posit that mutation of each S/T and/or D/E residue singly or in combination within these clusters should disrupt interactions with either one of these E2/E3 complexes and thus abrogate/attenuate the ubiquitination of the K-residue targeted within that cluster, thereby providing the required proof of concept.

Conceivably, surface complementarity with the interacting partners may also require positively charged residues in the neighborhood of the D-E-S/T-ubiquitinated K-residue clusters. Indeed, close scrutiny of the surface topology of the identified CYP3A4 and CYP2E1 phosphorylation and ubiquitination sites (Fig. 4) reveals yet another previously unappreciated structural feature: Many (but not all) of these residues lie within the same proximal P450 interface known to be important for protein-protein interactions with the P450 redox partners CPR and/or b_5 (90–93). Thus in addition to D-E-S/T clusters, these P450 interfaces include a series of basic (R/K) residues determined to be important for electrostatic interactions with corresponding acidic (E/D) residues of either the CPR/FMN domain or b_5 domain. These R/K residues are commonly thought to comprise the P450 "*positively charged bowl*" (40). Accordingly, on the basis of site-directed mutagenesis, homology modeling, and chemical cross-linking coupled with LC-MS/MS analyses, CYP3A4 residues K91, K96, K127, R130, S134, K421 and R446 have been identified as critical for electrostatic and/or H-bond interactions with CPR or b_5 (92, 93). Inspection of the CYP3A4 "*positively charged bowl*" harboring these particular basic residues (Fig. 4), reveals that some residues are themselves post-translationally modified (K127, S134) and/or lie in the close proximity to residues that are phosphorylated (T92, S100, S131, T136, S139, S420) (36). Similarly, not only were residues K428 and K434 in the b_5-interacting CYP2E1 interface (40, 90), ubiquitinated (35), but S424, S431 and T432 in their immediate vicinity were also PKC-phosphorylated (35). Thus, it appears that the post-translational modification (phosphorylation/ubiquitination) of some residues in the positively charged bowl would on one hand disrupt P450 interactions with its redox catalytic partners thereby abrogating its catalytic function, and rendering the P450 surface accessible to E2/E3 interactions. On the

other hand, similar modification of other residues (i.e. the phosphorylation of CYP3A4 S478) on a different P450 surface would enhance its recognition by E2/E3 ligases. These two concurrent processes would thus synergistically commit the P450 protein firmly to ERAD/UPD.

Collectively, our findings reveal that gp78 and CHIP E3-ligases play a vital role in hepatic P450 turnover and thus regulate its content and function, thereby influencing a myriad of physiologically, therapeutically and toxicologically relevant responses. In this, phosphorylation of P450 S/T residues within spatially associated D/E-surface clusters serves as a switch by generating "conformational acidic phosphodegrons" that mark the P450 proteins for ERAD/UPD. Phosphodegrons are known to be a common feature of substrate recognition by CHIP- and SCF (Skp1-Rbx1-Cul1-F-Box subunit complex)-E3 ligases (86, 87, 94). However, we believe our findings are the first to invoke a specific role for protein phosphorylation and "phosphodegrons" in gp78-substrate targeting. Whether protein phosphorylation is a common mechanism for gp78-recognition of its other substrates, and the precise gp78 structural domains involved in this recognition remain to be elucidated.

Acknowledgments

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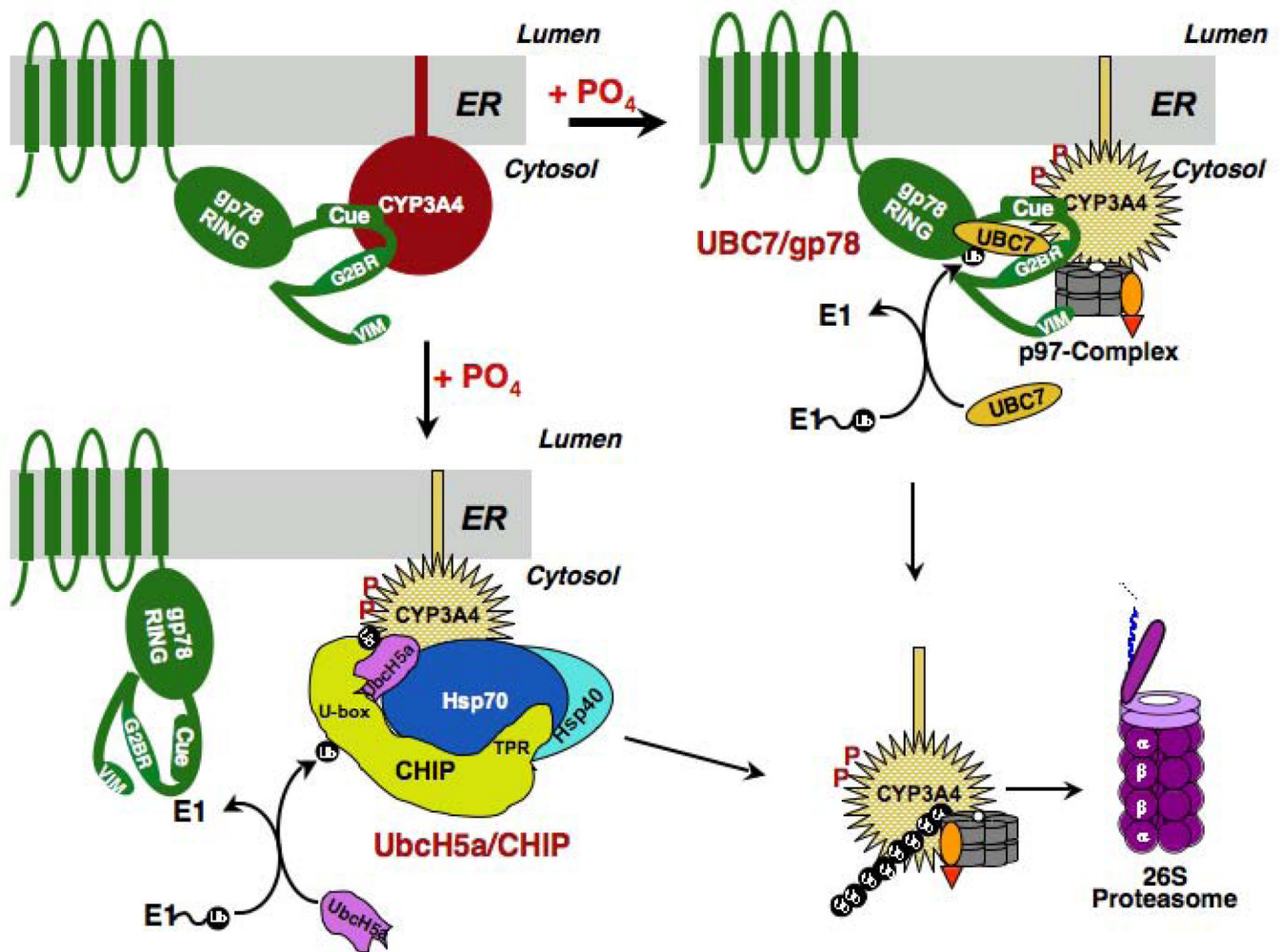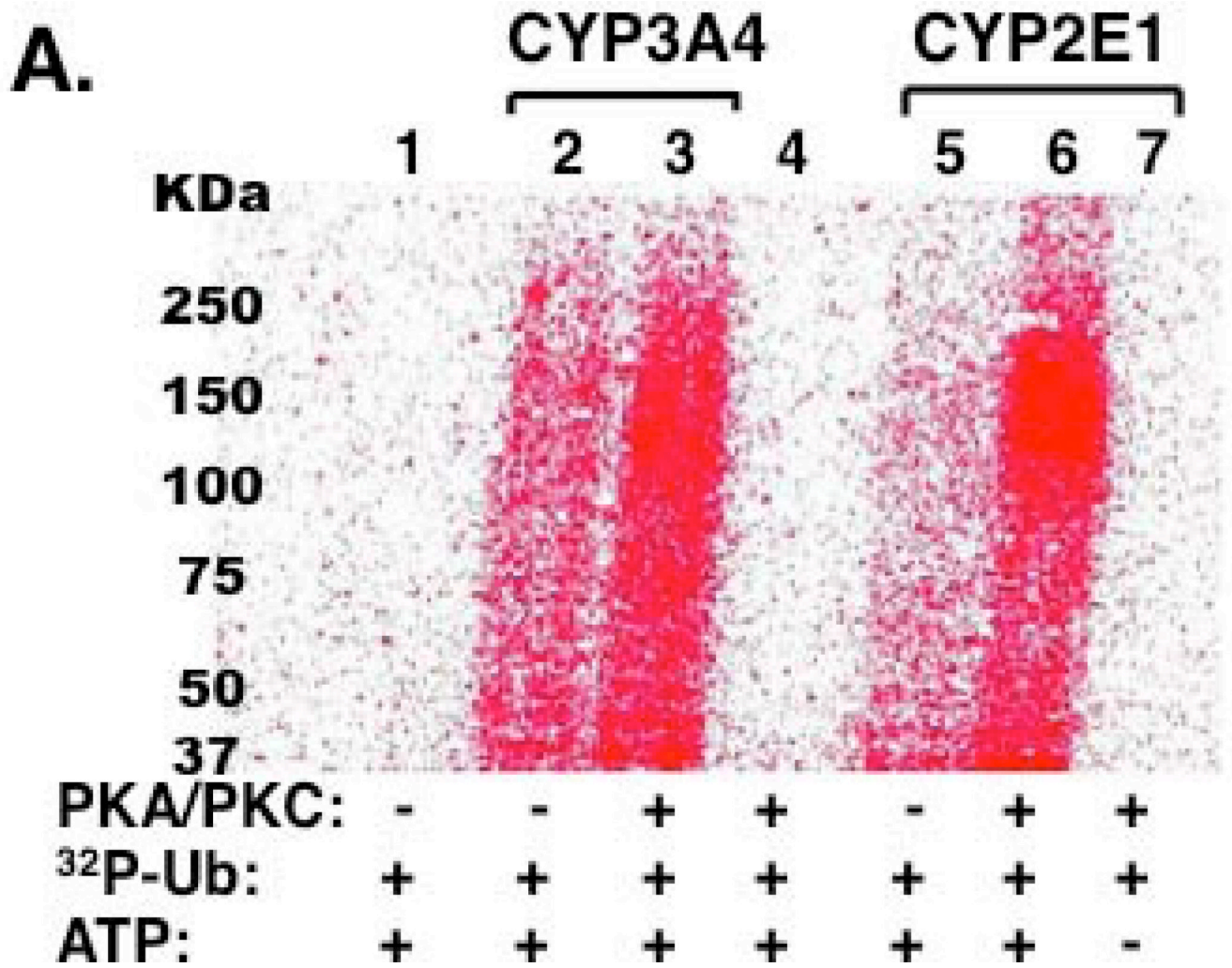
Fig. 1. CYP3A4 ERAD
Phosphorylation targets the ER-anchored P450 protein to ubiquitination by UBC7/gp78 and UbcH5a/CHIP/Hsp70/Hsp40 complexes and subsequent ER-extraction into the cytosol by the p97/Ufd1/Npl4 complex and 26S proteasomal degradation.
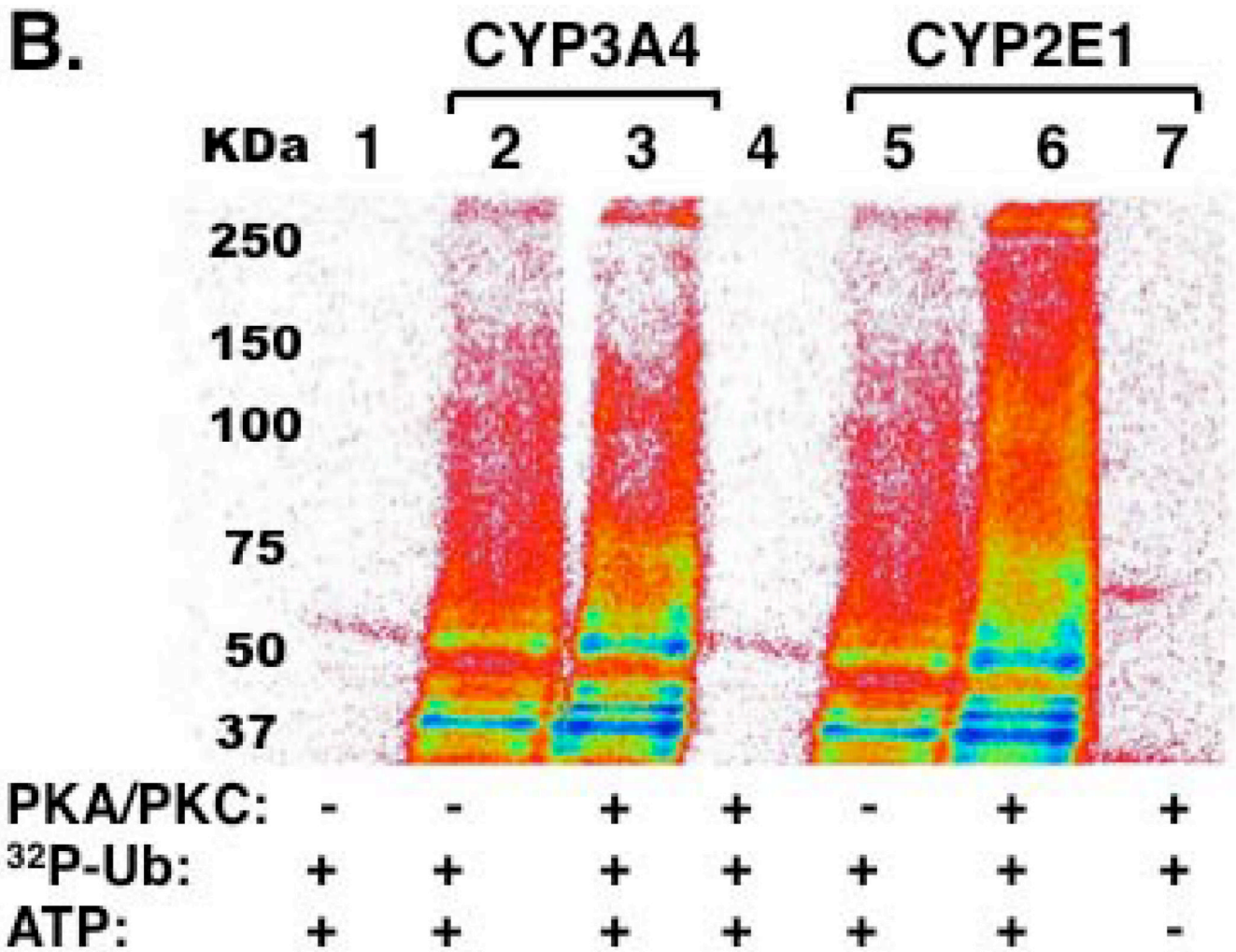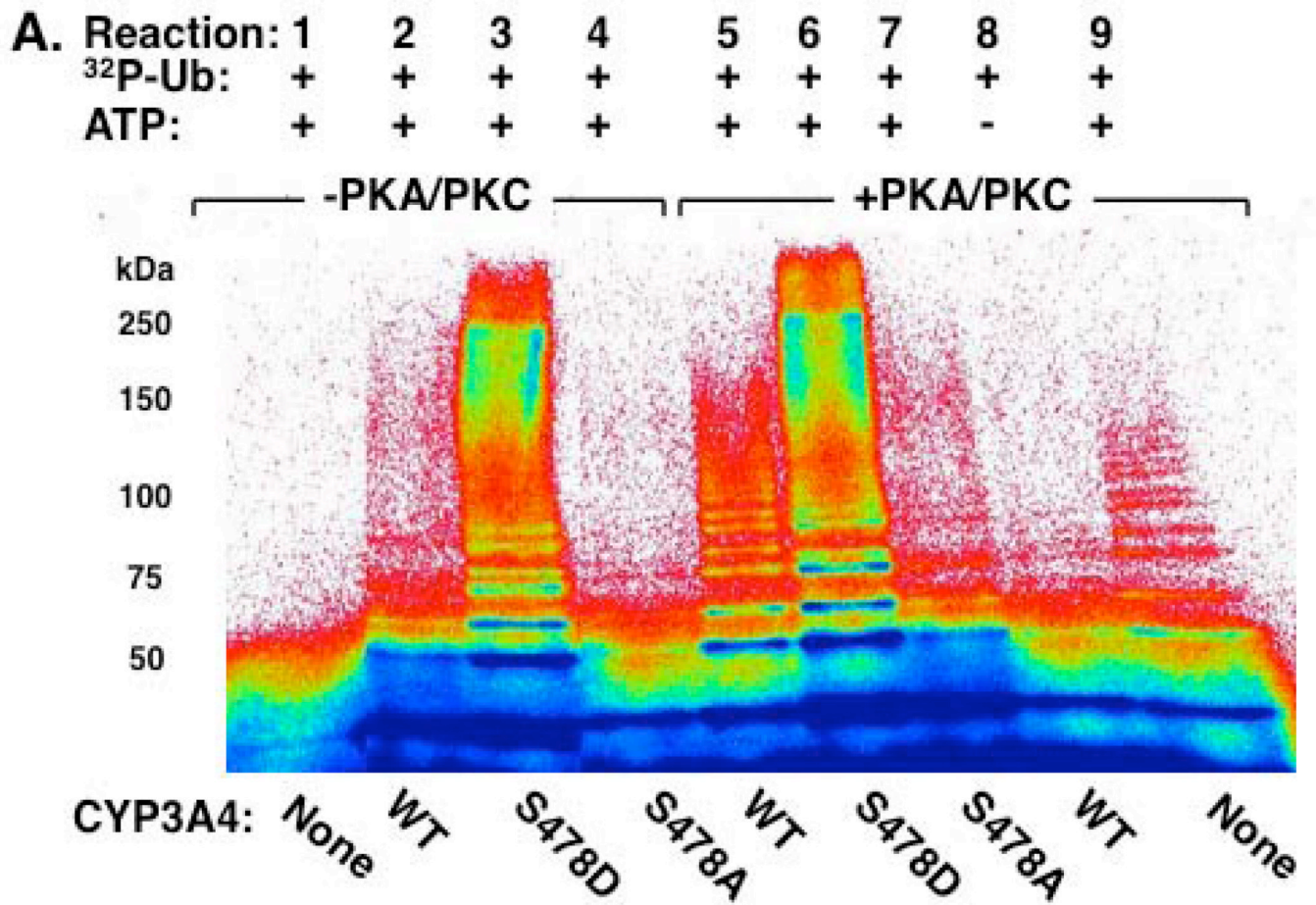
Fig. 2. Phosphorylation enhances the overall CYP3A4 and CYP2E1 in vitro ubiquitination by UBC7/gp78 and UbcH5a/CHIP/Hsp70/Hsp40 complexes
A. P450 ubiquitination by UBC7/gp78 in the presence and absence of PKA and PKC in a functionally reconstituted system incubated for 90 min. **B.** P450 ubiquitination by UbcH5a/CHIP/Hsp70/Hsp40 in the presence and absence of PKA and PKC in a functionally reconstituted system incubated for 90 min. For experimental details see Refs. 35 and 36.
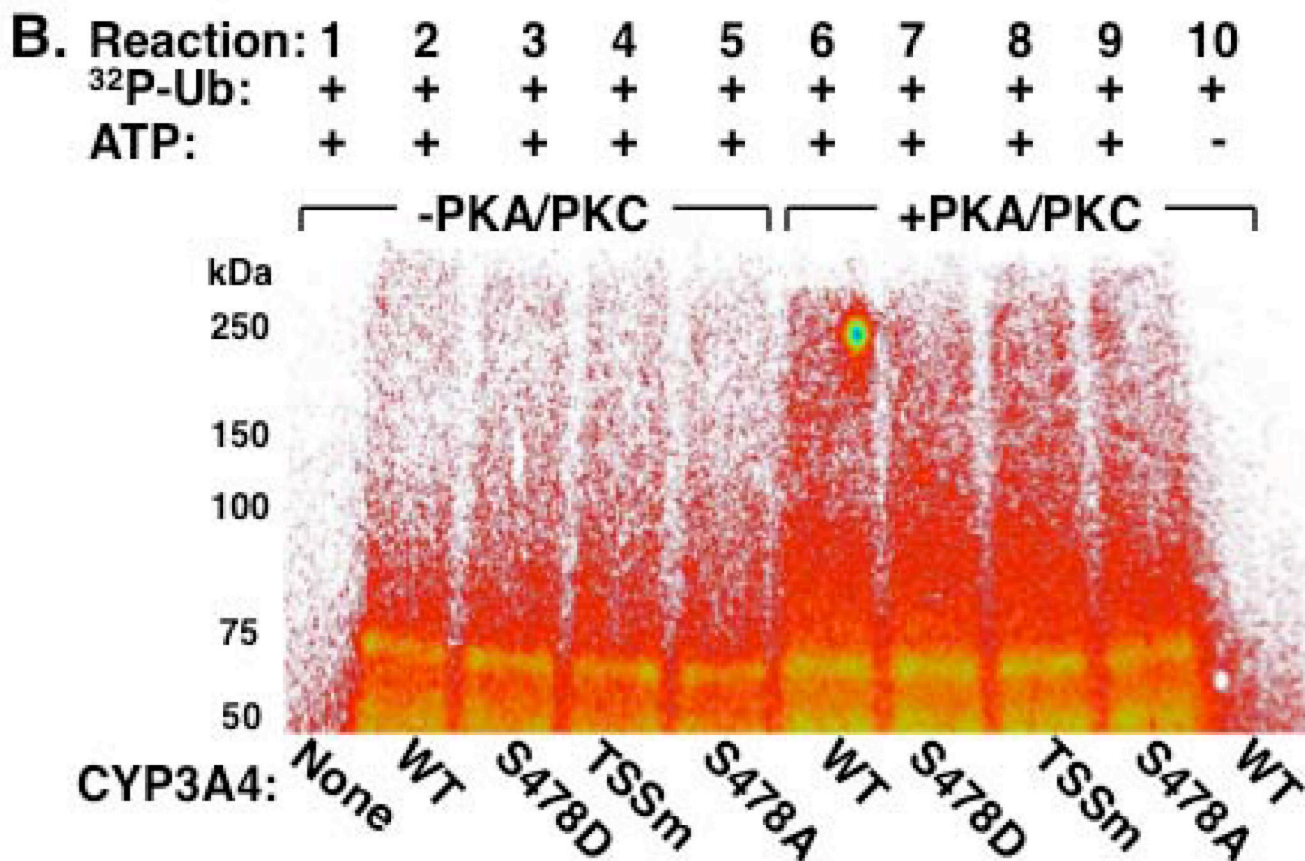
Fig. 3. The critical role of CYP3A4 S478 phosphorylation in its ubiquitination by UBC7/gp78 but not UbcH5a/CHIP/Hsp70/Hsp40 complexes

A. CYP3A4 wild type (WT), its S478D phosphomimetic mutant or its S478A phosphorylation impaired mutant was incubated for 90 min in an UBC7/gp78 functionally reconstituted system in the presence and absence of PKA and PKC as detailed (35, 36). **B**. Corresponding incubations of these CYP3A4 proteins in an UbcH5a/CHIP/Hsp70/Hsp40 functionally reconstituted system in the presence and absence of PKA and PKC as detailed (35, 36). TSSm is a triple CYP3A4 T264A/S420A/S478A mutant also included to determine the role of the other two residues that are substantially phosphorylated *in vitro*.
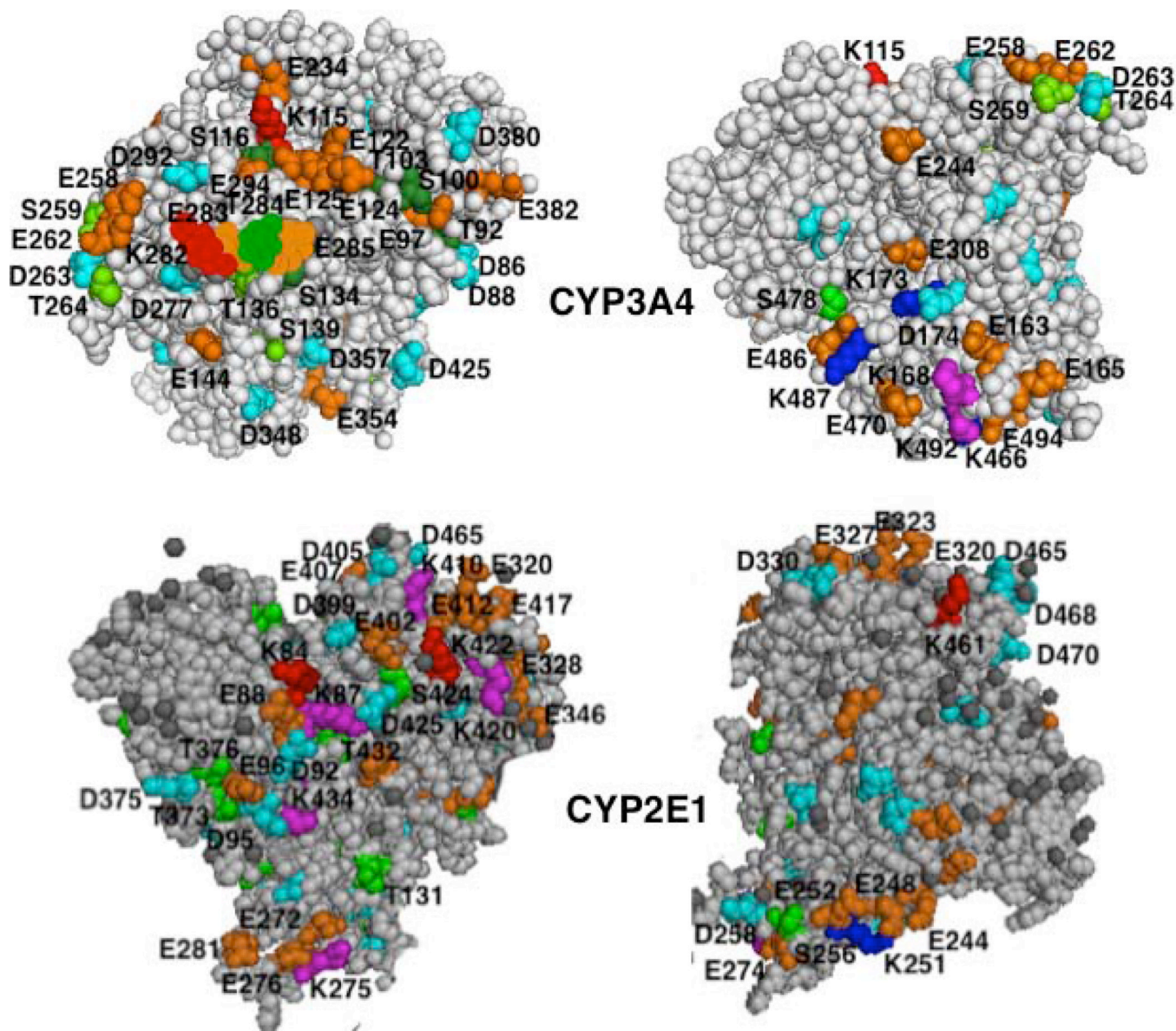
Fig. 4. CYP3A4 and CYP2E1 crystal structures: Conformational phosphodegrons
P450 S/T residues that we identified through LC-MS/MS analyses to be phosphorylated *in vitro* are shown in green (35, 36). D-residues are shown in cyan and E-residues in orange. K-residues identified to be ubiquitinated by UBC7/gp78 alone are shown in red, those ubiquitinated by UbcH5a/CHIP/Hsp70/Hsp40 in blue, and those by both E2/E3 complexes in magenta (35, 36). Two opposite surfaces of each P450 are depicted based on their reported crystal structures (40, 72–74).