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FOR THE RECORD

Novel domains in NADPH oxidase subunits, sorting nexins, and PtdIns 3-kinases: Binding partners of SH3 domains?

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Abstract: Two SH3 domain-containing cytosolic components of the NADPH oxidase, $p47^{phox}$ and $p40^{phox}$, are shown by analyses of their sequences to contain single copies of a novel class of domain, the PX (*phox*) domain. Homologous domains are demonstrated to be present in the Cpk class of phosphatidylinositol 3-kinase, *S. cerevisiae* Bem1p, and *S. pombe* Scd2, and a large family of human sorting nexin 1 (SNX1) homologues. The majority of these domains contains a polyproline motif, typical of SH3 domain-binding proteins. Two further findings are reported. A third NADPH oxidase subunit, $p67^{phox}$, is shown to contain four tetratricopeptide repeats (TPRs) within its N-terminal $Rac1^{GTP}$-binding region, and a 28 residue motif in $p40^{phox}$ is demonstrated to be present in protein kinase C isoforms ι/λ and ζ, and in three ZZ domain-containing proteins.

Keywords: homology; signal transduction; chronic granulomatous disease; tetratrico peptide repeats; phospholipase D

Patients with chronic granulomatous diseases (CGDs) are severely predisposed to infection by fungi and bacteria due to deficiencies of the component subunits of NADPH oxidase. Dysfunction of the oxidase reduces superoxide generation, thereby compromising a major non-specific host defense mechanism of phagocytes (reviewed in Segal, 1989). The most common cause of CGDs is an X-linked inheritance, resulting in the deficiency of the large β subunit of flavocytochrome *b* ($gp91^{phox}$). In approximately 30–40% of cases, however, autosomally inherited deficiencies of other subunits with masses 47k and 67k ($p47^{phox}$, and $p67^{phox}$) lead to CGD (Clark et al., 1989; Leto et al., 1990). Stimulation of neutrophils or other phagocytes results in these two cytosolic proteins, along with the small GTPases Rac1 or Rac2, translocating to the plasma membrane; subsequently, they form the functional NADPH oxidase, in complex with the transmembrane and heterodimeric $gp91^{phox}$-$p22^{phox}$ flavocytochrome b_{558}. This is the minimum complex required to generate the microbicidal superoxide anion. An additional cytosolic factor, $p40^{phox}$, is known also to associate with the NADPH oxidase complex (Wientjes et al., 1993), although its regulatory roles remain uncertain.

$P40^{phox}$, $p47^{phox}$, and $p67^{phox}$ each contain *Src* homology 3 (SH3) domains, which mediate multiple associations with proline-rich targets within the NADPH oxidase complex (Fuchs et al., 1995; de Mendez et al., 1996). These domains are also present in a diverse range of kinases, phosphatases, phospholipases, and cytoskeletal proteins, and bind left-handed polyproline type II helices (reviewed in Pawson, 1995). The presence of other domain types in $p47^{phox}$ and $p67^{phox}$, however, has not previously been noted. Here I report the identification of a novel domain family that includes $p40^{phox}$, $p47^{phox}$, phosphatidylinositol (PtdIns) 3-kinases, homologues of a sorting nexin (SNX1), and several yeast proteins, including the SH3-containing protein, Bem1p. I also record the presence of tetratricopeptide repeats (TPRs) in $p67^{phox}$, and note that a 28 residue (octicosapeptide) repeat (OPR), present in $p40^{phox}$ and other proteins (English et al., 1995), is also present in protein kinase C (PKC) ι/λ and ζ isoforms and three ZZ domain-containing proteins.

A continuing interest in C2 domain-containing proteins (Ponting & Kerr, 1996; Ponting & Parker, 1996) prompted Blastp (Altschul et al., 1994) comparisons with databases of a region of the Cpk class of PtdIns 3-kinases (MacDougall et al., 1995; Molz et al., 1996; Virbasius et al., 1996), which intervenes between their catalytic and C2 domains. These searches revealed moderate similarities within the N-terminal region of human $p47^{phox}$ (lowest probability of matching by chance, $p = 0.02$). Further evidence that Cpk-like PtdIns 3-kinases and $p47^{phox}$ contain an homologous domain was provided by SWise (Birney et al., 1996) database searches, which demonstrated $p47^{phox}$ to be the highest scoring sequence using a Cpk-derived profile. A subsequent Blastp search with the $p47^{phox}$ N-terminal sequence demonstrated additional similarities with regions of $p40^{phox}$ and orthologous *S. cerevisiae* and *S. pombe* sequences, Bem1p and Scd2 (p-values < 0.02). These three molecules also scored highest in SWise database searches using Cpk- and/or $p47^{phox}$-derived profiles. These results strongly suggest that these molecules contain an homologous domain, which I shall term the PX (*phox*) domain. This suggestion is compatible with previous observations of pairwise similarities between $p47^{phox}$ and Bem1p (Chenevert, 1994), and $p47^{phox}$ and $p40^{phox}$ (Wientjes et al., 1993).

Significantly, the top six highest scoring sequences in a SWise search using a subsequent profile (derived from Cpk, $p40^{phox}$,

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p47^{phox}, Bem1p, and Scd2p sequences) were human sorting nexin-1 (SNX1) and 5 *Saccharomyces cerevisiae* proteins, MDM1p, Mvp1p, D9461.13p, YDL113c, and P9513.1p. Using SWise, Blastp, and MACAW, each of these six was demonstrated to be a member of a 12-strong family of homologues (data not shown; Fig. 1a); previously, only SNX1 and Mvp1p had been proposed as homologues (Kurten et al., 1996). Multiple alignments (Schuler et al., 1991; Thompson et al., 1994) of SNX1-homologues showed that their regions of highest conservation coincided with their PX domain-like sequences. Indeed, motif searches employing the MoST algorithm (Tatusov et al., 1994) identified PX domain-like motifs in SNX1 homologues and, conversely, SNX1-like motifs in previously-defined PX domains (searches used α1 alignment blocks, *r*-values of 0.01 or 0.03, and parameter i = 80%); similar results were found using SWise (not shown). It is concluded that these SNX1-homologues also contain single copies of the PX domain.

In a final SWise database search, using a newly derived profile, four of the top seven top-scoring sequences were phospholipase D

Fig. 1. *See caption on facing page.*

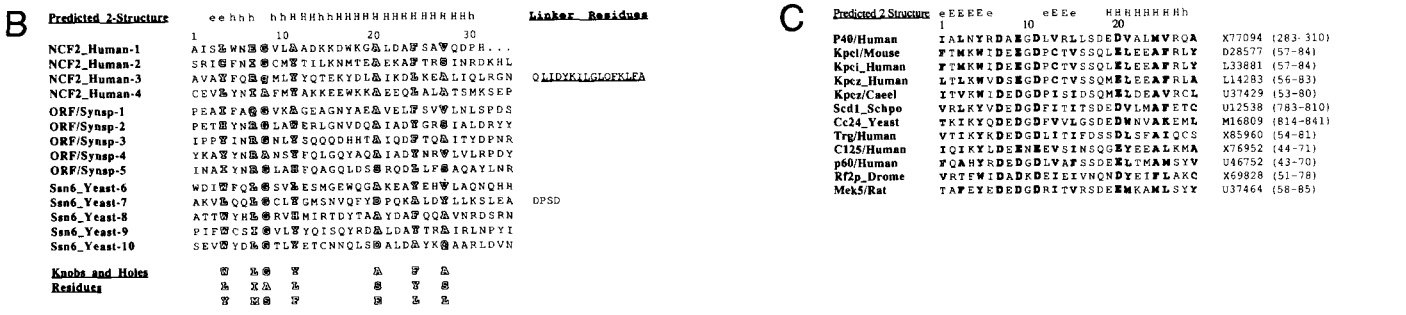

Fig. 1. (*Continued from facing page.*) Multiple alignments of (a) PX domain, (b) TPR, and (c) OPR sequences. Secondary structure predictions, calculated using PHD (Rost & Sander, 1994), are shown above the alignments [H/h denotes an α-helix and E/e a β-strand, predicted with expected accuracies of $\geq$82% (upper case) or otherwise $\geq$72% (lower case)]. EMBL database accession codes and residue limits are shown following the alignments, except in (b) [human p67^{phox}: M32011 (residues 6–154); *Synechocystis* sp. ORF: D10474 (61–230); and, *S. cerevisiae* Ssn6p: M23440 (224–397)]. (a) Alignment of PX domain sequences constructed using ClustalW (Thompson et al., 1994). Residues conserved in $\geq$60% of sequences are shown in outline; hydrophobic residues (A, C, F, I, L, M, V, and Y), present in $\geq$90% of sequences are in bold. Dots indicate insertions/deletions, whereas dashes indicate incomplete sequences. Intervening sequences are represented by numbers in parentheses. In closely similar paralogues (e.g., p47^{phox} and Cpk PtdIns 3-kinases) further sequence similarity is evident within regions N-terminal to the sequences shown here, implying the presence of (an) additional secondary structure(s); construction of accurate alignments in this region, however, was not possible. The C-terminal limit shown is coincident with the C-termini of Yor357c/Yeast and Yhq5_Yeast. The last 12 sequences represent translations of expressed sequence tags (ESTs); of these, some contain contributions from overlapping ESTs. (b) Four TPR motifs in p67^{phox}. An initial Blastp (Altschul et al., 1994) search using the human p67^{phox} sequence ("NCF2_HUMAN," residues 1–223) produced probabilities $p(n)$ of matching by chance over n alignment blocks of $p(2) = 6.5 \times 10^{-4}$ for *Synechocystis* sp. ORF248 and $p(2) = 3.1 \times 10^{-3}$ for *S. cerevisiae* Ssn6p; other TPR-containing sequences (e.g., *S. cerevisiae* OM70, human Cdc27, and *E. nidulans* BimA) were also represented as top scoring sequences. Four-way alignments of two p67^{phox} TPRs and either two *Synechocystis* sp. ORF248 or two *S. cerevisiae* Ssn6p TPRs produced typical p-values of 10^{-3}–10^{-5} (Schuler et al., 1991); aligning the four p67^{phox} TPRs produced a p-value of 3.7×10^{-5} (Schuler et al., 1991). Positions where amino acids form the hole (positions 4, 7, 8, and 11) and the knob (positions 20, 24, and 27) of TPRs are shown in outline; the three most frequent residues at these positions in TPRs (Sikorski et al., 1990) are also given. P67^{phox} TPR1 appears to contain only 31 residues, and a truncated TPR appears to follow TPR3 (underlined). The amino acid Gly$_{78}$ substituted in an autosomal recessive CGD (de Boer et al., 1994) is in lower case (p67^{phox} TPR3 position 8). (c) Alignment of p40^{phox}-like OPR sequences (conserved acidic residues shown in outline and hydrophobic residues conserved in all but a single sequence shown in bold). These sequences scored highly in a profile database search (Birney et al., 1996) with the addition of Ref(2)p, which appears to be an orthologue of p62B, a Lck SH2 domain-binding ligand. Four-way alignments of diverse OPRs yielded typical p-values of 10^{-7}–10^{-14} (Schuler et al., 1991), demonstrating that these sequence similarities are significant.

(PLD) orthologues from *H. sapiens*, *S. pombe*, *S. cerevisiae*, and *C. elegans* (cf. Ponting & Kerr, 1996). These similarities could not be demonstrated to be significant using MoST, SWise, or Blastp searches. However, three-way MACAW alignments of PLD1 orthologues with two PX domain-containing proteins revealed two alignment blocks with low probabilities of aligning by chance (e.g., for human PLD1, p47^{phox}, and SNX1, $p = 3 \times 10^{-3}$ and 1×10^{-2}). Assignments of PX domains in N-terminal regions of PLD1, therefore, are made tentatively, and further data are required to clarify this issue.

PX domains may possess SH3 domain-binding functions since it is noted that the majority of PX domains contain a polyproline motif X-P-p-X-P (where X is an aliphatic residue, particularly Ile or Leu, or else Met) within the polypeptide linking α-helices 1 and 2 (Fig. 1a). This motif is identical to the left-handed polyproline type II helices that bind many SH3 domains (reviewed in Pawson, 1995) and may also bind WW domains (Chan et al., 1996). Other potential ligands of PX domains that do not contain SH3 domains include Cdc24p (Petersen et al., 1994) and Ste20p (Leeuw et al., 1995), since these are known to bind Bem1p via regions that overlap with its PX domain (Fig. 2). The PX domains of SNX1 and Mvp1p have been suggested to bind vesicular trafficking machinery (Kurten et al., 1996), including perhaps dynamin homologues (Ekena & Stevens, 1995). In contrast to this potential role in vacuolar protein sorting, the PX domain in Vam7p may, instead, function by regulating *S. cerevisiae* vacuolar assembly (Wada & Anraku, 1992).

The presence of PX domains in SH3-containing proteins Bem1p, Scd2, and phox proteins provides an additional common feature in eukaryotic signalling pathways involving Rho-related GTPases. In *S. cerevisiae*, Bem1p binds to the putative scaffolding protein Ste5p and to the Rho-type GTPase Cdc42p, thereby linking GTPase activity with Ste20p, the kinase initiator of the mitogen-activated protein kinase (MAPK) cascade (Peterson et al., 1994; Leeuw et al., 1995; Lyons et al., 1996). A similar pathway has been shown in *S. pombe* (Chang et al., 1994). In humans, the scaffold is provided by the sum of NADPH oxidase subunits that bind both a GTPase, Rac1, and Pak, a human homologue of Ste20p (Diekmann et al., 1994; Manser et al., 1994; Prigmore et al., 1995). PX domain-containing PtdIns 3-kinases and PLD isoforms may further regulate the mammalian pathway via the activation of protein kinases that phosphorylate p47^{phox} (Ding et al., 1995; McPhail et al., 1995).

Examination of other regions of phox proteins using Blastp (Altschul et al., 1994) revealed two further observations. The N-terminal region of p67^{phox} was found to contain four imperfect copies of the TPR motif (Fig. 1b). These are 34 amino acid motifs, predicted to form amphipathic α-helices, that are thought to self-associate via a 'knob and hole' mechanism whereby a bulky hydrophobic residue (position 24; Fig. 1b), flanked by two small residues (positions 20 and 27), fits into a depression formed by residues at positions 4, 7, 8, and 11 (Sikorski et al., 1990). This is likely to account for the absence of the p67^{phox} protein in an autosomal recessive CGD patient who is homozygous for a mutation causing the amino acid change Gly$_{78}$ $\rightarrow$ Glu (de Boer et al., 1994). This residue maps to position 8 of TPR3, which is critical for formation of the non-polar 'hole.' Disruption of TPR self-association as a consequence of this mutation is likely to destabilize p67^{phox} folding and/or structure in a manner previously predicted for similar TPR position 8 Gly $\rightarrow$ Asp substitutions in temperature-sensitive nuc2 and CDC23 mutants (Hirano et al., 1990; Sikorski et al., 1993). The p67^{phox} TPRs (residues 6–154) are wholly contained within the Rac1^{GTP}-binding region (residues 1–199; Diekmann et al., 1994), suggesting that destabilization of TPRs results in abrogation of Rac1-mediated signalling.

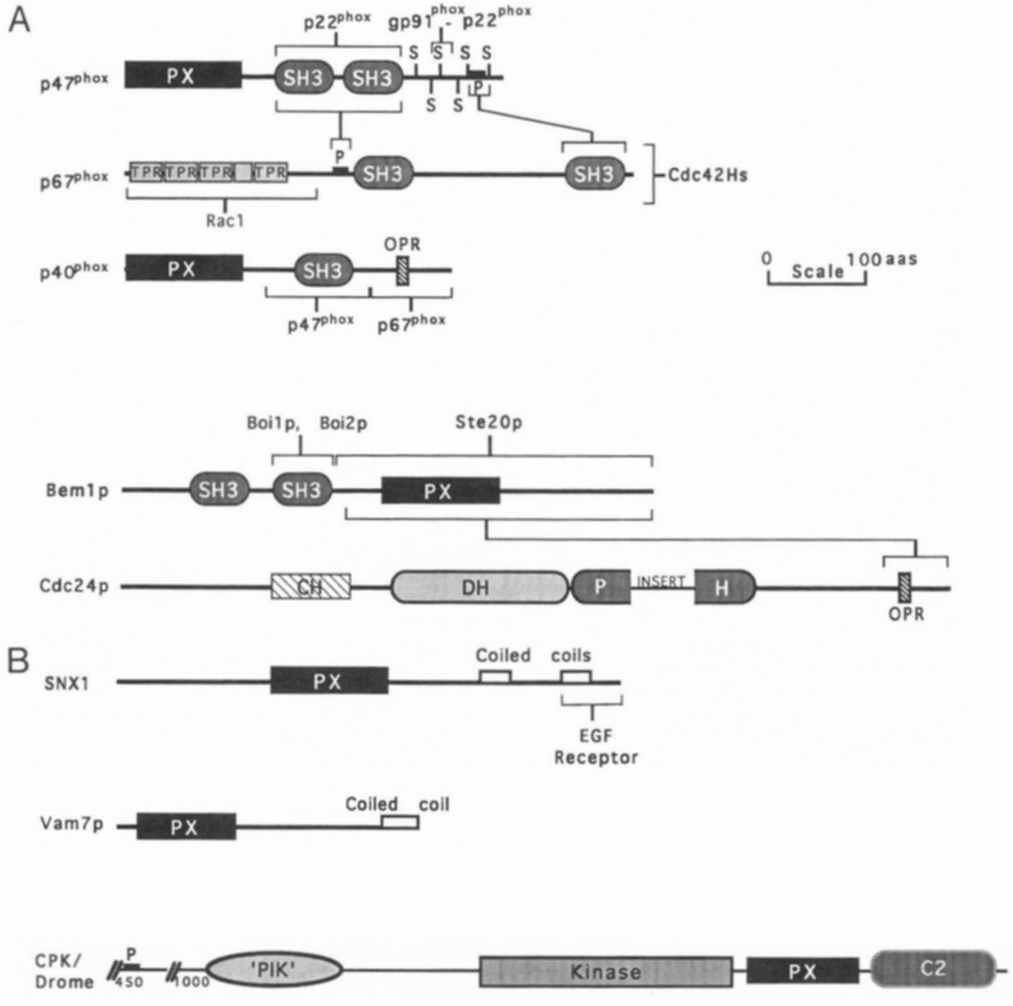

Fig. 2. The domain architecture of representative PX domain-, TPR-, and OPR-containing proteins. Abbreviations: DH, dbl-homologous domain; PH, (split) pleckstrin homology domain; PIK, a domain common to PI3Ks. (a) Phox subunits, Bem1p and Cdc24p. Protein–protein interactions, determined using domain deletion experiments (Diekmann et al., 1994; Petersen et al., 1994; deLeo et al., 1995; Fuchs et al., 1995; Bender et al., 1996; de Mendez et al., 1996; Matsui et al., 1996), are indicated by brackets; P denotes proline-rich regions; S denotes serine residues in $p47^{phox}$ that are phosphorylation targets of kinases. A putative calponin-homology (CH) domain (Castresana & Saraste, 1995) in Cdc24p has not been proposed previously. (b) Human SNX1, yeast Vam7p, and *Drosophila* Cpk. The majority of SNX1-homologues contain coiled coil regions, as predicted using COILS (Lupas et al., 1991). The C-terminal SNX1 coiled coil has been shown to bind the epidermal growth factor (EGF) receptor (Kurten et al., 1996).

$P40^{phox}$ and the Bem1p-binding protein, Cdc24p, each possess single OPR motifs, as do Scd2 and the MAP kinase kinase, MEK5 (English et al., 1995). Further searches (Altschul et al., 1994; Birney et al., 1996) using these as query sequences yielded similar motifs in atypical PKC ι/λ and ζ isoforms (Ono et al., 1989), a TRK-fusion gene product (Greco et al., 1995) and three ZZ domain-containing proteins, CA125, ref(2)P and p62, a $p56^{lck}$-binding protein (Dezelee et al., 1989; Campbell et al., 1994; Park et al., 1995; Ponting et al., 1996). The Bem1p-binding region of Cdc24p (residues 780–854; Petersen et al., 1994) contains its OPR (residues 814–841), suggesting that this function may be mediated by the OPR motif. Similarly, data (Fuchs et al., 1995) indicate that $p40^{phox}$ may bind $p67^{phox}$ via its OPR sequence (Fig. 2). OPRs, which do not fit the consensus sequence of EF-hands, possess four conserved acidic residues (positions 7, 9, 11, and 20; Fig. 1c), suggesting that these repeats bind divalent cations, such as Ca^{2+}. This is supported by observations that the interaction between Cdc24p to Bem1p is inhibited by Ca^{2+} (Zheng et al., 1995).

In conclusion, a novel domain was found to occur in two component subunits of NADPH oxidase ($p47^{phox}$ and $p40^{phox}$) and in a variety of other eukaryotic molecules; in addition, four TPRs were identified in the $Rac1^{GTP}$-binding region of $p67^{phox}$. The latter observation is likely to account for the molecular defect in a well-characterised case of CGD (de Boer et al., 1994). This contribution to the definition of domains in NADPH oxidase subunits is expected to facilitate characterization of their structures and functions, and the understanding of the molecular basis of autosomally inherited CGDs.

Note added in proof

Since completion of this work several PX domain-containing sequences have been deposited in databases (a human ORF, and *C. elegans* C05d9.1, F25h2.2, and F17h10.3). Using a revised alignment, profile methods now indicate the presence of PX domains in a second bud emergence protein, Bem3p, and in vacuolar protein

sorting-associated protein VPS17p. These sequences have been added to Figure 1A.

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