RESEARCH COMMUNICATION

Phage display identifies thioredoxin and superoxide dismutase as novel protein kinase C-interacting proteins: thioredoxin inhibits protein kinase C-mediated phosphorylation of histone

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Using phage display we identify the redox proteins thioredoxin and superoxide dismutase (SOD) as novel protein kinase C (PKC)-interacting proteins. Overlay assays demonstrated that PKC bound to immobilized thioredoxin, providing supporting evidence for the phage display results. Kinase assays demonstrated that SOD and thioredoxin were not direct substrates for PKC but that both proteins blocked autophosphorylation of PKC. Moreover, thioredoxin inhibited PKC-mediated phosphorylation of histone (IC_{50} of approx. 20 ng/ml).

Key words: binding protein, cell signalling, overlay assay, oxidative stress, phagemid.

INTRODUCTION

The protein kinase C (PKC) family of closely related phospholipid-dependent serine/threonine kinases is of fundamental importance in signal transduction processes [1,2]. Eleven subtypes of PKC with distinct enzymological characteristics and various intracellular locations have been identified [3]; these different PKC subtypes may have distinct and specialized functions in cell signalling [1,2]. PKC subtypes are subdivided into three main groups, the conventional (c) PKC subtypes α, β_I, β_{II} and γ, requiring calcium and diacylglycerol for activity, the novel (n) calcium-independent PKC subtypes δ, ϵ, η and θ, activated by diacylglycerol alone and the atypical (a) subtypes ζ, ι and λ, which are both calcium and diacylglycerol independent [3]. A wide range of substrate proteins are phosphorylated by PKC [4] and some such as myristoylated alanine-rich C-kinase substrate (MARCKS) have been well characterized.

Protein–protein interactions can be investigated by a number of techniques including yeast two-hybrid screens, interaction cloning or phage display [5]. In addition, biophysical methods for the detection of molecular interactions including surface plasmon resonance can verify postulated interactions [6]. In this study we have used the technique of phage display with a full-length liver cDNA library to identify putative PKC-interacting proteins using various PKC subtypes, as well as a short consensus peptide (Leu^{530}–Val^{558}) from the substrate binding region, as baits. We have chosen to identify PKC-interacting proteins using the phage display technique due to the high titre of phage libraries and the simplicity of the technique.

A number of phage display libraries displaying random peptides of full-length cDNA libraries are now commercially available. The gene protein 3 (gp3) technique has been utilized to display peptides and the products of full-length cDNA. This technique allows the display of proteins normally expressed by the tissue plus mRNAs which are translationally repressed [7]. Parmley and Smith [8] devised a simple procedure, termed 'biopanning', to affinity select for phage which display a peptide or protein that interacts with an immobilized substrate 'bait'. These 'baits' can be a short peptide to identify specific targeting sequences or a whole protein to identify substrates and/or regulatory proteins. Our studies have unexpectedly identified thioredoxin and superoxide dismutase (SOD) as interacting directly with PKC at the molecular level. These *in vitro* observations have been confirmed using an overlay method, while kinase assays show that thioredoxin and SOD inhibit PKC autophosphorylation and that thioredoxin can act as an inhibitor of PKC-mediated phosphorylation of histone.

EXPERIMENTAL

Materials

A phagemid display expression library (pEZM3) incorporating human liver cDNA was obtained from ClonTech (Basingstoke, U.K.) together with *Escherichia coli* TG1recO with the genotype: *supE thi*-1 Δ(lac-proAB) hsdΔ5[F′ traD36$^+$ proAB$^+$ lac^q lacZΔM15]. Purified PKC-α and PKC-ϵ were obtained from Calbiochem (Nottingham, U.K.) and a short PKC consensus fragment 530–558 with the sequence Leu-Leu-Tyr-Glu-Met-Leu-Ala-Gly-Gln-Ala-Pro-Phe-Glu-Gly-Glu-Asp-Glu-Asp-Glu-Leu-Phe-Gln-Ser-Lle-Met-Glu-His-Asn-Val-NH_2 [9] was obtained from Sigma–Aldrich (Poole, Dorset, U.K.). M13K07 helper phage was obtained from Invitrogen (Leek, The Netherlands). Biotin-labelling kits and high-capacity binding streptavidin-coated microtitre plates were from Boehringer Mannheim (Sussex, U.K.). Plasmid DNA purification kits were from Qiagen (Crawley, West Sussex, U.K.). For the kinase assays [γ-^{33}P]ATP (AH9968) was obtained from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). Unless otherwise stated all other reagents were from Sigma–Aldrich.

Immobilization of specific PKC subtypes and consensus fragment

Biotin-labelling of PKC-α, PKC-ϵ and the consensus fragment was carried out according to the manufacturer's instructions. Briefly, 20 μg of purified PKC or 1 mg of the consensus PKC fragment was resuspended in 1 ml of PBS, pH 7.4. D-Biotinoyl-ϵ-aminocaproic acid-*N*-hydroxysuccinimide ester (biotin-7-NHS)

Abbreviations used: PKC, protein kinase C; MARCKS, myristoylated alanine-rich C-kinase substrate; RACK, receptor for activated C kinase; SOD, superoxide dismutase.

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was added as detailed in the manufacturer's instructions and incubated for 2 h at room temperature with gentle stirring. Remaining non-reacted D-biotinoyl-ϵ-aminocaproic acid-*N*-hydroxysuccinimide ester was removed by gel filtration on a Sephadex G-25 column. The labelled protein was eluted and collected in 0.5 ml fractions. Recovered fractions were assayed for protein using the bicinchoninic acid method (Pierce Warriner, Chester, U.K.) to identify aliquots containing the labelled PKCs or consensus fragment. Aliquots (20 μl) of the appropriately labelled PKC were placed in three sets of wells on the streptavidin-coated plate and incubated for 1 h on an orbiting platform at room temperature. One set of wells was used for biopanning protocols and stored at 4 °C. A second set of wells was used to confirm immobilization of the appropriate ligand using anti-biotin and anti-(PKC subtype) specific antibodies [10] followed by chemiluminescent detection (results not shown).

Phage display protocol

A 10 μl aliquot of the original phage library (ClonTech) was diluted with 90 μl of blocking buffer [PBS, pH 7.4, 0.05 % Tween-20, 1 % (w/v) BSA] and applied to a PKC-α-, PKC-ϵ- or consensus fragment-coated well. The library was incubated in the well for 1 h at 37 °C. The well was then washed seven times with wash buffer (PBS, pH 7.4, 0.05 % Tween-20). Log-phase TG1 (100 μl) were then added to each panned well and incubated for 30 min at 37 °C and stored at −70 °C following the addition of 20 % (v/v) sterile glycerol.

A 50 μl aliquot from the phage library recovered from this first biopanning was added to 1 ml of 2 × YT medium [1 % (w/v) yeast extract, 1.7 % (w/v) tryptone, 0.5 % (w/v) NaCl] containing 2 % (w/v) glucose and incubated at 37 °C for 1 h with shaking. Ampicillin to a final concentration of 100 μg/ml was added followed immediately by 5×10^9 plaque-forming units of M13K07 helper phage i.e. a multiplicity of infection of approx. 20:1 (phage:bacteria). Cultures were then incubated at 37 °C for 1 h with shaking followed by centrifugation at 2000 ***g*** for 10 min at room temperature. The supernatant was discarded. The pellet was resuspended in 10 ml of 2 × YT supplemented with ampicillin (50 μg/ml) and kanamycin (25 μg/ml) and incubated overnight at 37 °C with shaking. The cultures were centrifuged at 10000 ***g*** for 20 min at 4 °C and the supernatant (which contains the enriched library) was transferred to a fresh tube. This centrifugation step was repeated to further clarify the culture.

Purification of the phage libraries was now carried out by adding 10 ml of blocking solution (PBS containing 0.05 % Tween-20 and 1 % BSA) to 10 ml of the enriched (crude) phage library followed by incubation at 4 °C for 10 min. Subsequently 4 ml of 20 % (w/v) sterile polyethylene glycol 8000 was added and the mixture incubated at 4 °C for 30 min to precipitate the phage. Phage particles were sedimented at 10000 ***g*** for 20 min at 4 °C, the supernatant was discarded and the pellet was resuspended in 1 ml of PBS. The resuspended pellets were transferred to sterile microfuge tubes and centrifuged at 13000 rev./min (max speed) in a microfuge for 10 min at 4 °C to remove the polyethylene glycol. The supernatants were retained. These purified, enriched phage libraries were then used for second and third rounds of biopanning as above.

After the third round of biopanning, 1 μl of recovered *E. coli* TG1recO from each library was diluted to 100 μl with 2 × YT and spread on to a 2 × YT/ampicillin/glucose/$MgCl_2$ plate and incubated overnight at 37 °C. Single colonies were picked and inoculated into 4 ml of 2 × YT/ampicillin/glucose and incubated overnight at 37 °C with shaking. Plasmid DNA was purified using Qiagen Mini-Prep kits, according to the manufacturer's instructions (Qiagen, Crawley, West Sussex, U.K.), followed by automated fluorescence DNA sequence analysis. DNA sequences were submitted to the SEQNET database for identification.

Overlay assay

Purified thioredoxin (1 mg) was biotinylated and added to wells of streptavidin-coated microtitre strips (Boehringer Mannheim). Wells were then blocked with 5 % (w/v) Marvel in TBS (25 mM Tris/HCl, pH 7.4, 0.15 M NaCl) containing 0.2 % Tween-20 for 1 h on a rocking table. Freshly passaged, overgrowing 3T6 fibroblasts were allowed to settle for 15 min to express PKC-ϵ as well as PKC-α, PKC-δ and PKC-ζ [11]. Attached cells were scraped into 100 μl of lysis buffer [10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5 % SDS, 1 % (v/v) Triton X-100, 1 % (w/v) deoxycholic acid]. This lysate was added to 3 ml of 1 % Marvel/TBS/Tween-20 which was dispensed as 100 μl aliquots into the thioredoxin-coated wells. Lysates were incubated in the wells for 2 h at room temperature on a rocking table. Wells were washed twice with TBS/Tween-20, once with 0.5 M NaCl and once more with TBS/Tween-20. Any PKC subtypes bound to the immobilized thioredoxin were detected by incubating the wells for 1 h at room temperature with a 1:1000 dilution of the appropriate PKC-specific antibody in 1 % Marvel/TBS/Tween-20. This was followed by incubation for 1 h with a 1:50000 dilution of secondary antibody in 1 % Marvel/TBS/Tween-20 and subsequent chemiluminescence detection [10]. The specificity of the immunodetection was confirmed by appropriate peptide knockouts.

PKC assays *in vitro*

The kinase assays were based on a method described by Yasuda et al. [12]. Briefly, 30 μg of phosphatidylserine and 0.3 μg of diolein (Gibco) per sample were air dried from a stock solution in chloroform. To this was added a reaction mix containing 2.5 mM Tris/HCl, pH 7.4, 5 mM MgAc and 0.5 mM $CaCl_2$ and made up to a reaction volume of 250 μl per sample with deionized H_2O. The reaction mix was sonicated for 10 min and vortexed to disperse the phosphatidylserine. Where appropriate, 0.1 unit of purified PKC and/or 50 μg histone was added to the reaction which was started with the addition of 25 μM ATP containing 0.1 μCi/reaction [γ-^{33}P]ATP. One unit of PKC is the amount required to transfer 1 nmol of phosphate to histone from ATP per min at 30 °C. Reactions were incubated at 30 °C with shaking for 30 min. In some experiments, increasing amounts of thioredoxin were included in the reaction. Proteins were precipitated by the addition of 2 ml of ice-cold 15 % (v/v) trichloroacetic acid following the addition of 100 μl of 1 % BSA to each reaction. Samples were kept on ice for 1 h and were then applied to 25 mm glass fibre filters under vacuum. The filters were washed with cold 15 % trichloroacetic acid followed by four further washes with 8 % trichloroacetic acid, which were then followed by two final washes with 80 % (v/v) ethanol. The filters were dried at 37 °C for 1 h. Trapped radioactivity was determined by placing the filters in scintillation vials with 2 ml of Ultima Gold XR scintillation fluid and measuring the counts on a liquid scintillation counter.

RESULTS

Library enrichment, analysis and the identification of interacting clones

The original phage library with a titre of 10^{10} colony forming units/ml represents a total of 5×10^8 viable individual phage

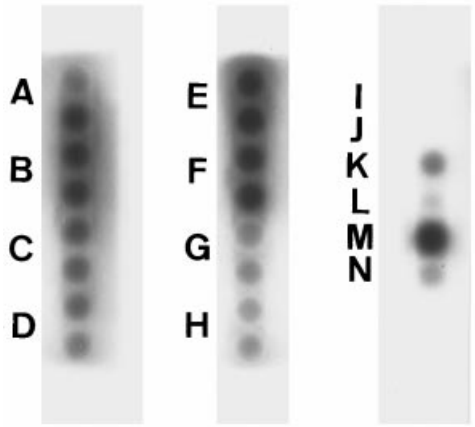

Figure 1 Overlay assay demonstrating that thioredoxin interacts with PKC-α, PKC-δ, PKC-ε and PKC-ζ

Duplicate wells coated with immobilized thioredoxin were probed with PKC-specific antibodies following incubation with cell lysates from newly passaged 3T6 fibroblasts. Lane A, PKC-α; Lane B, PKC-δ; Lane C, PKC-α Ab + peptide control; Lane D, PKC-δ Ab + peptide control; Lane E, PKC-ϵ; Lane F, PKC-ζ; Lane G, PKC-ϵ Ab + peptide control; Lane H, PKC-ζ Ab + peptide control. Control lanes I and J had no cell lysate added and were probed for PKC-α and PKC-ϵ respectively. Lanes K and L had cell lysate added but lacked immobilized thioredoxin; these were probed for PKC-α and PKC-ϵ. Lane M, immobilized thioredoxin was incubated with rat brain lysate and then probed for PKC-α. Lane N, peptide knockout.

particles per 50 μl aliquot that was used in the first biopanning. After successive rounds of biopanning and repeated washing the number of trapped phage particles retained by each substrate can be assessed by performing duplicate colony assays. Recovery of bound phage after the first round of biopanning was typically in the order of 0.0001 % of the original library i.e. approx. 500 individual phage per well.

Biopanning revealed a number of different PKC-interacting clones of which we have sequenced eighty to date. Fifty-two clones contained cDNA inserts whereas the remainder were deletion mutants. DNA sequence analysis confirmed that a number of the clones containing inserts displayed thioredoxin (three from ten clones after biopanning PKC-α, three from nineteen clones after biopanning PKC-ϵ and two from three clones from biopanning the consensus peptide fragment). Recovered clones contained sequences that had 83–99 % identity with thioredoxin. A single clone with an insert that had 91 % identity to the gene for SOD was identified which interacted with PKC-ϵ. In addition, ten clones, isolated without bait, were sequenced as examples of phage particles interacting non-specifically with streptavidin-coated plates. Five of these had sequences matching alpha-foetoprotein, three matched gamma globin and two were deletion mutants. These proteins were also detected binding non-specifically in several clones from the final biopannings across PKC-α and PKC-ϵ coated plates and are presumed to represent 'false positives' from the technique. No clones displaying thioredoxin or SOD were isolated within this control group.

Thioredoxin overlay assay

To confirm the validity of the interaction between thioredoxin and PKC, an overlay assay was employed. Commercially available thioredoxin (Sigma) was immobilized as described in the Experimental section and incubated with cell lysates from newly passaged 3T6 fibroblasts. Results from this approach show that

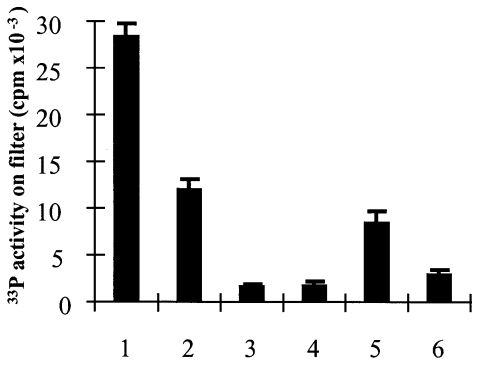

Figure 2 Thioredoxin blocks autophosphorylation of PKC

Both thioredoxin and MnSOD reduce the incorporation of ^{33}P into PKC to basal levels i.e. inhibiting autophosphorylation. Lane 1, ^{33}P incorporation in the presence of PKC and histone; lane 2, PKC alone (autophosphorylation); lane 3, 1 μg thioredoxin, no histone; lane 4, 10 μg thioredoxin, no histone; lane 5, 100 units (30 μg) of MnSOD, no histone; lane 6, 1000 units (300 μg) of MnSOD, no histone. Data represent the means ± S.D. for triplicate experiments.

PKC-α, PKC-δ, PKC-ϵ and PKC-ζ were detected bound to immobilized thioredoxin (Figure 1). The specificity of the antibody binding was demonstrated by its reduction following pre-incubation of antibody with the appropriate control peptide. Non-specific binding of the PKC antibody was not detected in the absence of cell lysates when the wells were probed for PKC-α and PKC-ϵ. However, some background was detectable when cell lysates were incubated with the wells in the absence of thioredoxin. PKC-subtypes from rat brain lysates (known to express high levels of PKC-α) interacted with immobilized thioredoxin in the same system (Figure 1). Figure 1 (lane A) has a reduced signal probably due to incomplete coating of the well with thioredoxin but the mean of the two PKC-α wells still remains above that of the peptide knockout as shown by laser-densitometry as follows. Mean laser-densitometry readings followed by the peptide knockout in brackets were: PKC-α,1.13 (0.85); PKC-δ, 1.19 (0.85); PKC-ϵ, 1.25 (0.82); PKC-ζ, 1.18 (0.74).

Thioredoxin and superoxide dismutase are not substrates for PKC but block its autophosphorylation

Thioredoxin and SOD were examined as possible PKC substrates in place of histone in kinase assays. Far from being phosphorylated and increasing the counts above PKC alone, incorporation of 1 and 10 μg/ml of thioredoxin and 100 (30 μg/ml) and 1000 units (300 μg/ml) of SOD into the assays reduced the counts detected on the filters to basal levels (Figure 2).

Thioredoxin is an inhibitor of PKC mediated phosphorylation of histone substrates

This effect was examined further by testing whether thioredoxin could inhibit the phosphorylation of histone by PKC. A range of thioredoxin concentrations from 1 ng/ml to 1 μg/ml in the kinase assay in the presence of 50 μg of histone inhibited the PKC-mediated phosphorylation of histone in a concentration-dependent manner with an IC_{50} of approx. 20 ng/ml, which is equivalent to 1.6 nM (Figure 3). Results are pooled data of duplicate samples from three experiments and show the thioredoxin-mediated inhibition of PKC activity relative to the control response of PKC and histone alone.

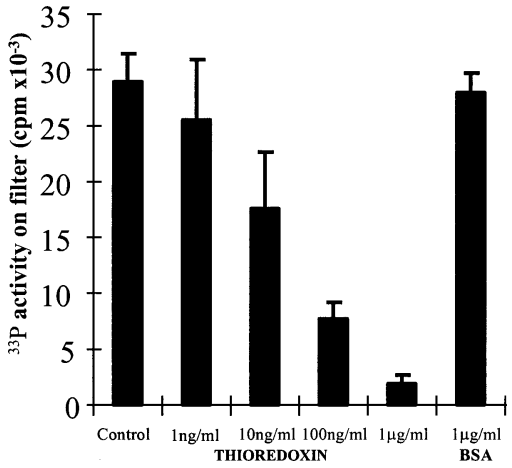

Figure 3 Thioredoxin inhibits PKC phosphorylation of histone

The addition of increasing amounts of thioredoxin (1 ng to 1 μg) to a PKC-mediated phosphorylation of histone reaction. BSA (1 μg) had no effect on PKC-mediated phosphorylation of histone. Data are the means $\pm$ S.D. for three separate experiments carried out in duplicate.

DISCUSSION

Phage display provides a powerful tool for the identification of protein–protein interactions. Phage libraries tend to have titres several orders of magnitude higher than that of yeast two-hybrid libraries enabling the detection of rare interactions. In addition, certain cytoplasmic interactions, that may not be detected in the yeast-two hybrid system where interactions occur in the nucleus, may be detectable by phage display. Nevertheless phage display has some drawbacks, most significant being that its interactions occur *in vitro* as opposed to within a cell. Further, some proteins may not insert into the inner membrane of the *E. coli* host or assemble correctly into a phage particle.

In the present study, phage display has identified thioredoxin as a novel potential substrate or regulatory protein interacting with PKC-α and PKC-ϵ. Furthermore, thioredoxin was the only protein identified to date that interacted with the 28 amino acid peptide derived from a PKC-consensus sequence (Leu^{530}–Val^{558}) from the substrate-binding region of the catalytic domain. This suggests that at least one site of interaction of thioredoxin with the intact PKC α and ϵ subtypes is at or near this substrate binding region. Another redox protein, SOD, was also identified as interacting with PKC-ϵ. The finding that PKC-α, PKC-δ, PKC-ϵ and PKC-ζ in fibroblast lysates bound to immobilized thioredoxin after extensive blocking and washing further confirms the validity of the phage display findings and extends the range of PKC subtypes that appear to interact with thioredoxin.

We were surprised that well-defined PKC interacting proteins such as MARCKS, receptor for activated C kinase (RACK), growth associated protein-43 (GAP-43), annexins, vinculin, perinuclear binding protein-1 (PICK-1) and adducin were not detected among the clones interacting with various PKC baits, whereas thioredoxin was identified in 25% of the clones sequenced. One reason may be that only a small fraction of the recovered libraries has been sequenced to date, 80 clones in total. So clones for MARCKS or RACK may be among remaining clones awaiting sequence. Another reason may be that the particular phage display library used was constructed from liver mRNA and thus proteins such as thioredoxin might be more commonly detected since they may be more highly expressed than MARCKS and RACK in liver. In addition, restriction analysis of the cDNA inserts (results not shown) indicated that the majority were approx. 1 kb, whereas the expected range indicated by the manufacturer was 0.3–3.0 kb. This means that full-length proteins with a molecular size below 40 kDa, or domains of larger proteins below this size, may dominate the library.

We have taken the phage display observations further to show that thioredoxin and SOD inhibit autophosphorylation of PKC. Furthermore, *in vitro* kinase assays show that thioredoxin inhibited the PKC-mediated phosphorylation of histone with an IC_{50} of 20 ng/ml (equivalent to 1.6 nM). How thioredoxin-mediated inhibition of PKC autophosphorylation occurs is unclear, although the fact that we identified clones displaying thioredoxin interacting with the consensus peptide suggests that binding between PKC and thioredoxin is partly in this region, which is quite close to the key, initial phosphorylation site (Thr^{500}) on the activation loop of PKC [13]. Phosphorylation of this site is a prerequisite for subsequent phases of autophosphorylation and activation of the kinase. Indeed, replacement of this residue with a non-phosphorylatable valine residue blocks activation of the kinase [14]. Thus we speculate that the binding of the 12 kDa thioredoxin protein close to this site may sterically block subsequent autophosphorylation and activation of PKC. This may explain our observation that thioredoxin blocks phosphorylation of histone by PKC (see Figure 3). Additionally, thioredoxin could inhibit histone phosphorylation by PKC by binding close to the catalytic region and preventing substrate–enzyme interaction.

Thioredoxin is a 12 kDa protein that has oxidoreductase activity via its S_2-$(SH)_2$ active site [15]. Oxidized thioredoxin is reduced by an NADPH-dependent thioredoxin reductase. Thioredoxin is present in the cytoplasm of all cells and is secreted from virus-infected cells [16]. It has a wide range of cellular functions concerned with hydrogen donation, free radical scavenging, protein disulphide reduction and protein folding. This is the first report of a direct interaction between PKC subtypes and thioredoxin and this *in vitro* finding with the phage display technique must now be confirmed in cells. Indirect evidence suggests that such an interaction might exist *in vivo* [17], since thioredoxin was rapidly translocated into the nucleus when HeLa cells were treated with the phorbol ester 12-O-tetradecanoyl phorbol 13-acetate (TPA) which, as a diacylglycerol analogue, activates many PKC subtypes. Thioredoxin itself has no reported authentic nuclear localization signal [17] but translocation of activated PKC subtypes into the nucleus is well documented [18]. Our finding of a thioredoxin–PKC interaction *in vitro* suggests the possibility that thioredoxin might be transported into the nucleus in association with a PKC subtype after phorbol ester activation. Other literature reports reveal that thioredoxin and PKC function in closely related pathways. For example, thioredoxin-SH2 promotes proliferation of human B-cell lines through a PKC-dependent mechanism [19]. However, this work does not indicate a direct interaction between PKC and thioredoxin in cells since the exogenously added thioredoxin activates phosphoinositide-specific phospholipase C and phospholipase D activities generating diacylglycerol and increasing intracellular calcium resulting in activation of PKC. The plasma membrane receptor for exogenous thioredoxin is not known but it is suggested [19] that plasma membrane thioredoxin reductase may function as a 'receptor' for extracellular thioredoxin. PKC is implicated in regulating expression of thioredoxin reductase since this is stimulated by phorbol ester treatment of thyrocytes [20].

Phorbol ester treatment of cells is widely reported to generate the production of reactive oxygen species in several cell systems [21–23]. The role of PKC in this process is not clearly defined. PKC-β has been implicated in signalling for oxygen radical generation in differentiated HL60 cells while superoxide production in human neutrophils might involve more than one PKC subtype [24]. PKC itself, like thioredoxin and thioredoxin reductase, contains redox-active cysteine-rich regions; those in the catalytic domain of PKC subtypes are sensitive to selenite and other selenocompounds which inactivate the kinase [25,26]. Thioredoxin along with thioredoxin reductase and NADPH acts as a disulphide reductase system protecting cells against oxidative stress. This system may act to combat the generation of potentially harmful reactive oxygen species linked to activation of PKC and our finding of a direct interaction between thioredoxin and PKC *in vitro*, where thioredoxin can block autophosphorylation and thus activation of PKC, reveals a new mechanism whereby thioredoxin might produce a protective effect against oxidative stress generated by activation of PKC.

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