The combination of H₂O₂ and vanadate generates aqueous peroxovanadium (pV) species, which are effective cell-permeable oxidants, and potent inhibitors of protein-tyrosine phosphatases. As a result, treatment of intact cells with pV compounds significantly enhances protein Tyr phosphorylation. Here we demonstrate that treatment of intact rat hepatoma Fao cells with pV markedly enhances Tyr phosphorylation of a 75-kDa protein, termed pp75. Amino-terminal sequencing of pp75 revealed that this protein is a member of the 70–75-kDa heat shock protein family, which includes PBP-74, glucose-related protein (GRP)-75, and mortalin. Tyr phosphorylation of pp75 is selective, because other proteins that belong to the heat shock protein 70 family, such as GRP-72, Bip (GRP-78), and HSC-70 fail to undergo Tyr phosphorylation when cells are treated with pV. Our findings suggest that heat shock proteins such as pp75 may undergo tyrosine phosphorylation when intact cells are subjected to oxidative stress induced by pV compounds.

H₂O₂ and vanadate mimic several of the metabolic and growth-promoting effects of insulin and related growth factors (1–5). In combination, their actions are strongly synergistic due to the formation of aqueous peroxovanadium (pV)¹ species (6, 7). Incubation of cells with pV leads to enhanced protein-tyrosine phosphorylation (1–3, 6, 8–9) due to activation of protein-tyrosine kinases (PTKs), which is secondary to inhibition of protein-tyrosine phosphatase (PTP) activity (3, 6, 10–12). In fact, pV compounds are the most potent inhibitors of PTPs yet described. Because pV compounds are known oxidants that have been shown to be much more effective than vanadate in oxidizing cysteines (13), their mode of action presumably involves oxidation of a cysteine residue present within a well-conserved sequence at the active site of all PTPs (14). In that respect, pV compounds differ from vanadate, which inhibits PTP activity by acting as a phosphate analog (15). Because pV compounds are easily accessible to the cellular interior (3), incubation of cultured cells with pV or its administration into rats increases severalfold the phosphorylation level and state of activation of several PTKs, including the insulin receptor β-subunit (insulin receptor kinase) (7, 12). This results in the phosphorylation of insulin-dependent in vitro substrates (7, 16), including insulin receptor substrate 1 (IRS-1) (17). IRS-1, which bears no enzymatic activity, is an adapter protein that Tyr phosphorylation of which creates binding sites for SH2-containing proteins, such as the p85 regulatory subunit of phosphatidylinositol 3-kinase (16, 18), GRB2, Nck, and SHPTP2 (Syp) (19).

We have shown previously (12, 16) that as a result of treatment of cultured cells or perfused rat livers with pV, not only IRS-1 but additional tyrosine-phosphorylated proteins are found in IRS-1 immunoprecipitates (16). The latter could be either proteins that are tightly associated with IRS-1 or proteins that cross-react with IRS-1 antibodies. Here we demonstrate that a protein, termed pp75, undergoes Tyr phosphorylation in response to treatment of cultured rat hepatoma (Fao) cells with pV. Moreover, pp75, which is selectively precipitated by antibodies directed against the carboxy-terminal end of IRS-1 (c-IRS-1), is a member of the heat shock protein (hsp) family.

The heat shock proteins, also known as molecular chaperones, help guide protein transport and folding under physiological conditions and limit proteotoxicity (aggregation and aberrant folding) during stress (20–23). hsps are grouped into several families according to their molecular weights (24, 25). In humans, the hsp70 multigene family consists of at least four members: hsc70 (26), hsp70 (27, 28), GRP-78 (BiP) (29), and hsp75 (30–33). The latter protein, having a molecular mass of ~75 kDa, has been independently cloned by several groups and was referred to either as PBP-74, a mouse protein implicated in B-cell peptide processing (31); mthsp75 (or GRP-75), a rat and human mitochondrial protein (30, 32); or as mortalin, a senescence-related gene product (33). pp75 described here, which is homologous to mthsp75 and presumably represents its hepatic murine isoform, is the first heat shock protein known to undergo tyrosine phosphorylation within intact cells.

MATERIALS AND METHODS

**Ligand Treatment of Intact Cells—**Rat hepatoma cells (Fao) were grown in RPMI 1640 containing 10% fetal calf serum. Confluent monolayers were deprived of serum for 16 h before treatment. Cells were then washed and incubated in serum-free medium. H₂O₂ and sodium orthovanadate were added either alone or in combination (3) to generate pV compounds (7). Insulin or other ligands were added to the medium as indicated. After incubation, cells were washed twice with ice-cold PBS and frozen in liquid nitrogen or immediately extracted in buffer I (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 10 mM sodium pyrophosphate, 2 mM Na₃VO₄, 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μM/ml aproasin, and 5 μg/ml leupeptin, pH 7.6). Extracts were centrifuged 30 min at 4 °C at 12,000 × g. The supernatants were mixed with concentrated (5 ×) sample buffer (34), run on 7.5% SDS-gels, and transferred to nitrocellulose papers for

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¹ The abbreviations used are: pV, peroxovanadium; PTK, protein-tyrosine kinase; PTP, protein-tyrosine phosphatase; IRS, insulin receptor substrate; GRP, glucose-regulated protein; hsp, heat shock protein; PAGE, polyacrylamide gel electrophoresis.

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Western blot analysis.

Subcellular Fractionation—Subcellular fractionation was carried out essentially as described by Graham (35). Briefly, rat livers (5 ml/g) or cultured Fao cells homogenates were prepared in buffer II (25 mM Tris, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 5 μg/ml leupeptin, pH 7.5) and centrifuged at 3000 x g for 10 min at 4 °C. The pellets were discarded, and the supernatants were further centrifuged at 12,000 x g at 4 °C for 30 min. The pellets (P1) were washed twice by resuspension in buffer II and centrifugation at 12,000 x g as above. The supernatants were centrifuged at 100,000 x g for 1 h at 4 °C. The final high-speed supernatant represents the cytosolic fraction, whereas the 100,000 x g total microsomal pellet (P2) was washed twice in buffer II. Final resuspension of P1 and P2 fractions was done in buffer II in the presence of 1% Triton X-100. Samples from each fraction were resolved by SDS-PAGE and transferred to nitrocellulose papers for Western blot analysis.

Antibodies—Affinity-purified Tyr(P) antibodies were generated as described previously (16). Antisera to insulin receptor substrate 1 (IRS-1) (36), termed c-IRS-1 antibodies, were raised in rabbits according to standard procedures (37), by injection of a peptide CYASIN-FQKQPEDRQ corresponding to the carboxyl-terminal 14 amino acids of rat liver IRS-1 (and an additional Cys residue at the amino-terminal site). Authenticity of the peptide was confirmed by sequencing. Antibodies against GRP-72 (38) and HSC-70 (26) were a generous gift of Dr. L. Pahl (National Institutes of Health, Bethesda, MD). Bip (GRP-78) (29) antibodies were kindly provided by Dr. M. Oren (Weizmann Institute).

Immunoprecipitation—Antibodies were added to 60 μl of 50% protein A-Sepharose solution in a buffer composed of 0.1 M Tris, pH 8.5, and were incubated for 1 h at 4 °C. The complex was precipitated at 12,000 x g (for 5 min) and washed three times in the incubation buffer. The last wash was in the sample’s extraction buffer. Tissue or cell extracts were prepared in extraction buffers as indicated. Five hundred-μl extracts (0.8 mg of protein) were incubated for 2 h with the antibody–Protein A-Sepharose complex. After incubation, the immunocomplexes were precipitated at 12,000 x g (for 5 min) and were washed four times with the extraction buffer. The final pellets were then suspended in Laemmli sample buffer (34), boiled for 5 min, resolved by 7.5% SDS-PAGE under reducing conditions (34), and transferred into nitrocellulose papers for Western blotting with the appropriate antibodies. Immunoblotting was performed as described previously (16), using an ECL kit purchased from Amersham Corp. and horseradish peroxidase-conjugated protein A, according to the manufacturer’s instructions.

Two-dimensional Gel Electrophoresis—Separation of proteins by two-dimensional gel electrophoresis was performed as described (39).

Amino-terminal Sequencing of Purified pp75—pp75 was immunoprecipitated by affinity-purified c-IRS-1 antibodies, and its amino-terminal sequence was determined as described by Ulrich et al. (40).

RESULTS

Specificity of c-IRS-1 Antibodies—Western immunoblotting of liver extracts with polyclonal antibodies directed against the carboxyl-terminal end of IRS-1 (c-IRS-1) did not reveal the presence of IRS-1 itself. Instead, the antibodies reacted with a protein having a molecular mass of 75 kDa, termed pp75. Binding of c-IRS-1 antibodies to pp75 was specific and could be abolished in the presence of the c-IRS-1 peptide (Fig. 1, lanes 2–3), indicating that pp75 contains an IRS-1-related antigenic epitope. Although c-IRS-1 antibodies failed to react with endogenous IRS-1 in total liver extracts, they did react, by means of Western immunoblotting, with IRS-1 derived from cells that overexpress the protein (41). Moreover, Tyr-phosphorylated IRS-1 was readily precipitated by c-IRS-1 antibodies (41) (Fig. 2). These findings suggested that although the content of IRS-1 in liver extracts is below the detection level of the c-IRS-1 antibodies, the antibodies react with pp75, which presumably contains an antigenic epitope corresponding to the carboxyl-terminal end of IRS-1.

Tyr Phosphorylation of pp75—Consistent with our previous studies (2, 8), incubation of Fao cells with insulin resulted in enhanced Tyr phosphorylation of IRS-1, present in c-IRS-1 immunoprecipitates (Fig. 2). Such treatment failed to induce Tyr phosphorylation of pp75 (Fig. 2). However, when Fao cells were incubated with a combination of H2O2 and vanadate (which generates pV compounds (7)), Tyr phosphorylation of both pp75 and IRS-1, present in IRS-1 immunoprecipitates, was readily detected (Fig. 2, top). The kinetics of pV-induced Tyr phosphorylation of pp75 and of IRS-1 were very similar. Phosphorylation of both proteins was rapid; it was half maximal by 3–5 min and reached its maximal levels following 10 min incubation with the oxidant (Fig. 2, bottom).

Characterization of pp75—Experiments were carried out to determine whether pp75 represents a degradation product of...
IRS-1. When hepatic pp75 was resolved by two-dimensional gel electrophoresis, it migrated with a calculated pI of ~6.5 (Fig. 3). This was in contrast to the calculated pI value of the carboxyl-terminal 735 amino acids of IRS-1, which is 8.0. In contrast to IRS-1, which upon cellular fractionation localizes to the 12,000 × g supernatant (42, 43), subcellular fractionation of rat liver demonstrated that pp75 was localized mainly to the 12,000 × g particulate fraction (Fig. 4).

The majority of Tyr residues within IRS-1, which are subject to phosphorylation, are located at the carboxyl-terminal 735 amino acids of the protein (17). Thus, in response to insulin stimulation, tyrosine phosphorylation of both IRS-1 and its putative degradation product pp75 should take place. We found, however, that only Tyr-phosphorylated IRS-1, but not pp75, was present in c-IRS-1 immunoprecipitates derived from insulin-treated Fao cells. Tyr phosphorylation of pp75 was, however, readily detected once Fao cells were treated with pV (cf. Fig. 2).

To determine the tissue distribution of pp75 and compare it with that of IRS-1, extracts from various rat tissues were resolved by SDS-PAGE, transferred to nitrocellulose papers, and Western immunoblotted with c-IRS-1 antibodies (Fig. 5). pp75 was found to be expressed to a comparable level in several tissues (i.e. spleen, pancreas, brain, kidney, liver, heart, muscle, fat, and testis) except for lungs. Furthermore, pp75 could not be detected in bacterial and yeast cell extracts (data not shown), indicating its specific expression in multicellular organisms. This pattern of tissue distribution differed from the expression pattern of IRS-1 mRNA (44).

Collectively, these experiments already indicated that pp75 is presumably not a degradation fragment of IRS-1; however, a better proof was obtained when the amino-terminal part of pp75 was sequenced.

**Amino-terminal Sequencing of pp75—Four μg of pp75 were immunoprecipitated with c-IRS-1 antibodies, transferred to polyvinylidene difluoride membrane, and were subjected to amino-terminal sequencing. When compared to the data bank, the sequence of 14 amino acids (Fig. 6A) revealed high homology (80%) to amino acids 47–60 of PBP-74 (31), GRP-75 (32), and mortalin (33), the three homologues of hsp75. Because it has already been demonstrated (31) that the translated product of hsp75 is cleaved at amino acid 46 to generate the mature protein, pp75 could represent a murine hepatic mature form of hsp75.

Because pp75 reacted with IRS-1 antibodies that were raised against a synthetic peptide corresponding to 14 amino acids located at the carboxyl-terminal end of IRS-1, we looked for the presence of similar antigenic epitopes within the sequences of hsp75. The consensus 10a amino acids at the carboxyl-terminal end of hsp75 is KQEDQKEEKQ. This stretch of charged residues shares 60% identity and overall 70% homology to the corresponding carboxyl-terminal 10 amino acids of IRS-1, which were part of the synthetic peptide used as antigen to generate the IRS-1 antibodies (Fig. 6B). Hence, being a member of the hsp75 family, the carboxyl-terminal end of pp75 could serve as an antigenic epitope to the c-IRS-1 antibodies. Of note is the fact that no such homologies were found when the sequence of IRS-1 was compared with other members of the hsp70 family (e.g. GRP-72 (38) or HSC-70 (26)), and indeed, c-IRS-1 antibodies failed to react with these proteins (Fig. 7).

**Selective Tyr Phosphorylation of pp75 but not of Other Members of the Hsp70 Family—**Once pp75 was identified as a putative heat shock-related protein that undergoes Tyr phosphorylation in response to oxidative insult, attempts were made to determine whether it undergoes Tyr phosphorylation in response to other forms of stress. However, pp75 failed to undergo enhanced Tyr phosphorylation when subjected to heat shock or pH changes (data not shown). To determine whether other members of the hsp70 family are also Tyr phosphorylated in response to oxidative stress, three additional heat shock proteins were precipitated from extracts of pV-treated Fao cells and blotted with Tyr(P) antibodies (Fig. 7). In contrast to pp75, GRP-72, BiP, and HSC-70 failed to undergo Tyr phosphorylation in response to pV treatment. This occurred in spite of the fact that significant amounts of these proteins were precipitated by their corresponding antibodies (Fig. 7).

**DISCUSSION**

In the present study, we demonstrate that oxidative stress, inflicted upon Fao cells in the form of pV, induces a rapid

![Image](http://www.jbc.org/)

**FIG. 3. Separation of pp75 by two-dimensional gel electrophoresis.** Total liver extract (200 μg) was resolved by two-dimensional gel electrophoresis, transferred onto nitrocellulose paper, and Western immunoblotted with affinity-purified c-IRS-1 antibodies. The location of pp75 is indicated by the arrow.

**FIG. 4. Subcellular distribution of pp75.** Liver extracts were fractionated and directly resolved by 7.5% SDS-PAGE (four left lanes). Alternatively, extracts were fractionated, immunoprecipitated with c-IRS-1 antibodies, and subjected to SDS-PAGE (four right lanes). Following transfer to nitrocellulose paper, Western immunoblotting was carried out with c-IRS-1 antibodies.

**FIG. 5. Tissue distribution of pp75.** Rat tissues were homogenized in buffer II, and 50 μg of protein sample from each tissue were subjected to SDS-PAGE, transferred to nitrocellulose papers, and Western immunoblotted with c-IRS-1 antibodies. Lane 1, spleen; lane 2, pancreas; lane 3, brain; lane 4, kidney; lane 5, liver; lane 6, heart; lane 7, muscle; lane 8, lungs; lane 9, fat; lane 10, testis.
tyrosine phosphorylation of a 75-kDa protein that belongs to the family of hsp70. Although pp75 was originally identified as a Tyr-phosphorylated protein present in IRS-1 immunoprecipitates, several lines of evidence were presented to suggest that pp75 is not a degradation product of IRS-1, nor is it an IRS-1-associated protein. Instead, pp75 has an IRS-1-related antigenic epitope that corresponds to the carboxyl-terminal 10 amino acids of IRS-1.

(i) Analysis of the isoelectric point of pp75 by two-dimensional gel electrophoresis indicated that pp75 migrates with a pI of 6.5, which differs from the calculated pI of 8.0 of the carboxyl-terminal 735 amino acids of IRS-1. (ii) Unlike IRS-1, which localizes to the cytosol and low-density microsomal fractions (43, 45), pp75 mainly associates with the high density (12,000 × g pellet) microsomal fraction. (iii) Unlike IRS-1, pp75 fails to undergo insulin-dependent Tyr phosphorylation, in spite of the fact that the carboxyl-terminal 735 amino acids of IRS-1 contains most of its insulin-stimulated Tyr phosphorylation sites (17). (iv) Sun et al. (44) have demonstrated that a 9.5-kb mRNA fragment is the major mRNA species encoding IRS-1. Although two smaller mRNA species of 1.4 and 2.2 kilobases were variably detected, these could not be translated into a 75-kDa protein because they do not include the 3′-end portion of the IRS-1 gene (44). Thus, pp75, most likely, is not an alternative splice variant of IRS-1, nor is it a degradation product of IRS-1.

Similarly, although pp75 is precipitated by c-IRS-1 antibodies, it is not an IRS-1-associated protein for the following reasons: (i) pp75 is not precipitated by other IRS-1 antibodies directed against a synthetic peptide corresponding to amino acids 489–504 of rat IRS-1 (16); and (ii) when total cell extracts are resolved by SDS-PAGE, pp75 directly interacts with c-IRS-1 antibodies by means of Western blotting. Collectively, these findings support our notion that pp75 contains an antigenic epitope that is selectively recognized by c-IRS-1 antibodies.

Sequencing of pp75, present in c-IRS-1 immunoprecipitates, revealed that its amino-terminal 14 amino acids is high homologous (80%) to amino acids 47–60 of mortalin (33), PBP-74 (31), GRP-75 (32), and mthsp75 (30). These four homologous proteins are encoded by the same cDNA and collectively represent the fourth family member of hsp70 proteins, termed hsp75. Because the translated product of hsp75 is cleaved at amino acid 46 to generate the mature protein (31), pp75 most likely represents the mature form of a hepatic member of the hsp75 family. Moreover, the consensus sequence of 10 amino acids at the carboxyl-terminal end of hsp75, QKEDQKEEKQ, shares 60% identity and overall 70% homology to the corresponding carboxyl-terminal 10 amino acids of IRS-1 that were part of the synthetic peptide used as antigen to generate the IRS-1 antibodies. Hence, the carboxyl-terminal end of pp75 presumably serves as an antigenic epitope to the c-IRS-1 antibodies. Because no other stretches of sequence homology between IRS-1 and hsp75 were found, it might explain the selective immunological cross-reactivity of pp75 with antibodies directed against the carboxyl terminus, but not other regions, of the IRS-1 molecule. In general, the hsp70 family members are more conserved at their amino-terminal ATP-binding domain and less conserved at their carboxyl-terminal end (25). This difference might account for the fact that c-IRS-1 antibodies, which react with antigenic epitope located at the carboxyl-terminal end of hsp75, fail to react with other members of the hsp70 protein family. Indeed, no sequence homologies are found between IRS-1 and hsp75. Members of the hsp70 family (e.g., GRP-72 (38) or HSC-70 (26)) are resolved by SDS-PAGE. Hence, the consensus sequence of 10 amino acids at the carboxyl-terminal end of hsp75, QKEDQKEEKQ, shares 60% identity and overall 70% homology to the corresponding carboxyl-terminal 10 amino acids of IRS-1 that were part of the synthetic peptide used as antigen to generate the IRS-1 antibodies. Because no other stretches of sequence homology between IRS-1 and hsp75 were found, it might explain the selective immunological cross-reactivity of pp75 with antibodies directed against the carboxyl terminus, but not other regions, of the IRS-1 molecule. In general, the hsp70 family members are more conserved at their amino-terminal ATP-binding domain and less conserved at their carboxyl-terminal end (25). This difference might account for the fact that c-IRS-1 antibodies, which react with antigenic epitope located at the carboxyl-terminal end of hsp75, fail to react with other members of the hsp70 protein family. Indeed, no sequence homologies are found between IRS-1 and other members of the hsp70 family (e.g., GRP-72 (38) or HSC-70 (26)). Collectively, these findings suggest that pp75 is presumably hepatic rat hsp75. Members of the hsp70 family exhibit different cellular localization. Whereas GRP-75 and mthsp75 (30) are localized to the mitochondria (32), PBP-74 is localized to cytoplasmic vesicles (31), and mortalin is found either in the cytosol or perinuclear region (46). The reason why
different homologues of the same protein exhibit different subcellular localization is presently unknown. However, due to the fact that pp75 is found mainly in the 12,000 × g pellet fraction, which is enriched with mitochondria, it seems reasonable to assume that pp75 resembles GRP-75 and mthsp75, which are mitochondrial proteins.

hsps are involved in all aspects of protein folding and oligomerization. In addition, hsps function in the intracellular transport of proteins to appropriate destinations, the disassembly of oligomeric structures, and facilitation of the removal of aggregated or improperly folded polypeptides (20, 22–25, 47). Although the general features of these activities are well documented, less is understood about the molecular mechanisms that regulate hsp function. Moreover, whereas stress-activated protein kinases or c-Jun amino-terminal kinases are known to be stimulated in response to a variety of stresses including heat shock (48, 49), hsps themselves are not considered targets for protein phosphorylation. hsp27 and hsp90 are the only hsps that have been reported to undergo phosphorylation (on Ser/Thr residues) (50–52). hsp27 is an oligomeric phosphoprotein, constitutively expressed in most human cells, whose phosphorylation in response to stress modulates actin filament dynamics (51), whereas hsp90 is subjected to Ser/Thr phosphorylation when cells are treated with the protein phosphatase inhibitor okadaic acid (52).

Our results suggest that pp75, the hepatic homologue of hsps, undergoes in vivo tyrosine phosphorylation when cells are subjected to oxidative stress upon incubation with pV compounds. This phenomenon seems to be selective because other proteins belonging to the hsp70 family, which include HSC-70 (26), Bip (53), and GRP-72 (38), fail to undergo Tyr phosphorylation under these conditions. Although the molecular consequences of hsp75 phosphorylation are presently unknown, Tyr phosphorylation is a key regulatory element in numerous signal transduction pathways and cellular processes (54). Moreover, a growing number of intracellular signaling molecules, receptors, kinases, and transcription factors are found associated with hsps (20, 55). For example, hsp90 associates transiently with pp60 v-src, directs its cellular trafficking, and negatively regulates its kinase activity (52). Moreover, Ser/Thr phosphorylation of hsp90 and/or pp60 v-src functions as a regulatory molecular trigger to release pp60 v-src from the chaperone complex at the inner surface of cell membranes (52). Taken together, these findings and our present results suggest that phosphorylation of hsps, either on Ser/Thr or on Tyr residues, might modulate their function and their mode of interaction with other cellular constituents.

pp75 undergoes Tyr phosphorylation when cells are treated with pV compounds. These agents, which act as intracellular oxidants (13), are the most potent inhibitors of PTPs, the activity of which depends upon the presence of reactive cysteine present within their active site (14). Inhibition of PTPs activity is, therefore, expected to indirectly enhance the basal activity of PTKs, which is maintained even in the complete absence of a ligand (3, 6, 8). This low level of PTK activity generates a cycle in which Tyr(P) is formed and degraded in the basal state at rates that prevent any significant net autophosphorylation. Upon administration of pV compounds, this equilibrium is disrupted because dephosphorylation is inhibited, allowing the low level of autophosphorylating activity to prevail, and leads to PTK activation. Therefore, PTKs that are stimulated through autophosphorylation like those belonging to the insulin receptor (2, 3, 8) or the Src family (56, 57) are expected to maintain a high kinase activity in pV-treated cells, even in the absence of a ligand. The nature of the pV-stimulated PTKs that phosphorylate pp75 is presently unknown; however, the possibility that pp75 is associated with mitochondrial-enriched fractions suggests that mitochondrial PTKs (58) might catalyze this reaction.

In conclusion, our results indicate that pp75, a member of the hsp70 protein family, undergoes tyrosine phosphorylation in response to pV treatment. This is the first evidence for tyrosine phosphorylation of a heat shock protein, which suggests that this posttranslational modification might regulate the function of pp75 or related hsps in a manner as yet to be defined.

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p75, a Member of the Heat Shock Protein Family, Undergoes Tyrosine Phosphorylation in Response to Oxidative Stress
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