Molecular Neurobiology

ISSN0893-7648/03/28(2):123–138/$25.00

Light Regulation of the Insulin Receptor in the Retina

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Abstract

The peptide hormone insulin binds its cognate cell-surface receptors to activate a coordinated biochemical-signaling network and to induce intracellular events. The retina is an integral part of the central nervous system and is known to contain insulin receptors, although their function is unknown. This article, describes recent studies that link the photobleaching of rhodopsin to tyrosine phosphorylation of the insulin receptor and subsequent activation of phosphoinositide 3-kinase (PI3K). We recently found a light-dependent increase in tyrosine phosphorylation of the insulin receptor-β-subunit (IRβ) and an increase in PI3K enzyme activity in isolated rod outer segments (ROS) and in anti-phosphotyrosine (PY) and anti-IRβ immunoprecipitates of retinal homogenates. The light effect, which was localized to photoreceptor neurons, is independent of insulin secretion. Our results suggest that light induces tyrosine phosphorylation of IRβ in outer-segment membranes, which leads to the binding of *p85* through its N-terminal SH2 domain and the generation of PI-3,4,5-P_3. We suggest that the physiological role of this process may be to provide neuroprotection of the retina against light damage by activating proteins that protect against stress-induced apoptosis. The studies linking PI3K activation through tyrosine phosphorylation of IRβ now provide physiological relevance for the presence of these receptors in the retina.

Index Entries: Insulin receptor; phosphoinositide 3-kinase; ROS, rod outer segments; phosphatidylinositol-4,5-bisphosphate; phosphatidylinositol-3,4,5-trisphosphate; platelet-derived growth factor; phototransduction.

Introduction

Communication between cells and their environment is of particular importance in living organisms. In eukaryotic cells, transmembrane signaling is accompanied by a variety of conserved mechanisms. Each mechanism uses a different strategy to circumvent the barrier posed by the lipid bilayer of the plasma membrane. Some signals are sufficiently lipid-soluble to cross the plasma membrane and act on intracellular receptors, which may be an enzyme or a regulator of gene transcription *(1)*.

Received 2/17/03; Accepted 2/17/03.
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Some bind to ligand-gated transmembrane ion channels and directly regulate the opening and closing of these channels *(2).* Others bind to transmembrane receptors, and trigger conformational changes in the receptors *(3).* The change in receptor structure activates the intrinsic enzymatic activity of the receptor *(4)* or other enzymes that are linked to the cytoplasmic domains of the receptor, which in turn activate different intracellular signaling cascades *(5).* The ligand-receptor binding may also activate guanosine triphosphate (GTP)-binding proteins (G proteins), which generate intracellular second messengers *(6).* A signal can propagate rapidly throughout the cell through the diffusion of these second messengers. A cell can interact with and respond to many different types of external factors, including hormones, neurotransmitters, chemicals, metabolites, other cells, and the extracellular matrix (ECM). Changes in the local concentration of these extracellular factors can elicit a remarkably diverse array of cellular responses, ranging from changes in motility *(7),* protein trafficking *(8),* and the cytoskeletal arrangement *(9)* to cellular gene expression and progression through the cell cycle *(10).* The precise response depends on the combination and the duration of activation of the intracellular pathways *(11).* Among these pathways, growth factor-activated signaling cascades have received particular attention, because they have been implicated in tumorigenesis. The growth-factor receptors are polypeptides, each consisting of an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic enzymatic domain, which may be a protein tyrosine kinase *(12)* or a serine kinase *(13).* Different receptors activate different intracellular effectors through phosphorylation or direct protein–protein interactions, and can result in cell survival or apoptosis, proliferation, or differentiation *(4).*

Growth Factors

In mammals, insulin is the principal hormone that controls blood glucose levels, and acts by stimulating glucose influx and metabolism in muscle and adipocytes and inhibiting gluconeogenesis by the liver *(14).* In addition, insulin modifies the expression or activity of a variety of enzymes and transport systems in nearly all cells. Insulin action is mediated through the insulin receptor, a transmembrane glycoprotein *(15)* that, like the receptors for epidermal growth factor (EGF) and platelet-derived growth-factor (PDGF) receptor, contains intrinsic tyrosine kinase activity *(16).* The level of tyrosine kinase activity reflects the serum concentration of insulin and appears to mediate the insulin response through autophosphorylation of the receptor itself and phosphorylation of substrates such as insulin receptor substrate-1 (IRS-1) *(17).* Insulin receptor autophosphorylation stimulates tyrosine kinase activity, but—unlike the EGF and PDGF receptors—the insulin receptor does not usually directly associate with SH2 proteins *(14),* although in a few studies, insulin receptors have directly associated with the SH2 group of the *p85* subunit of phosphoinositide 3-kinase (PI3K) *(18).* The activated insulin receptor usually phosphorylates IRS-1, a principal substrate of the insulin receptor, on multiple tyrosine residues, which in turn recognizes and binds to the SH2 domain of various signal-transduction proteins *(17).* In almost all studies to date, PI3K is activated when phosphorylated IRS-1 binds to the SH2 domains in its *p85*α regulatory subunit, which establishes a direct molecular connection between circulating insulin and this cellular enzyme *(19).* Other SH2 proteins, including SH-PTP-2 *(20),* GRB-2 *(21),* and Nck *(22),* associate with IRS-1 to mediate the pleiotropic insulin response.

Insulin Receptors

The insulin receptor is present in virtually all vertebrate tissues, although the concentration varies from as few as 40 receptors on circulating erythrocytes to more than 200,000 receptors on adipocytes and hepatocytes *(14).* The receptor gene is located on the short arm of human chromosome 19, is more than 150 kilobases in length, and contains 22 exons, which encode a 4.2-kb cDNA *(23).* The insulin receptor is composed of two α-subunits that are each linked to

a β-subunit, and to each other by disulfide bonds *(14).* Both subunits are derived from a single proreceptor by proteolytic processing at a cleavage site consisting of four basic amino acids. There is one site of alternative splicing surrounding exon 11, which results in two receptor isoforms that differ by 12 amino acids near the COOH terminus of the α-subunit *(23).* The mature heterotetramer (α2β2) contains complex N-linked carbohydrate side chains capped by terminal sialic acid residues, and migrates with a molecular mass of 300–400 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoreses (SDS-PAGE). The α-subunits are located entirely outside of the cell and contain the insulin-binding site(s), and the intracellular portion of the β-subunit contains the insulin-regulated tyrosine kinase *(14).*

Retinal Insulin Receptors

Cells of the bovine retina contain specific, high-affinity receptors for insulin *(24,25).* Studies of the receptor structure revealed the existence of two insulin-receptor subpopulations, with apparent mol wts of the α-subunits of 120 and 133 kDa *(26).* These receptors are in contrast to brain (122 kDa) and liver (133 kDa), in which a single α-subunit type was found. In addition, the retinal insulin receptors can be distinguished by their solubility in Triton X-100, glycosylation, and recognition by anti-insulin-receptor antibody *(26).* Despite these structural differences, the two populations of retinal receptors appear to have similar insulin-binding affinities. Examination of human retinas with site-specific antibodies to the C-terminus of human IRα has revealed two populations with apparent mol wts of 115 kDa and 125 kDa *(26).* However, a single heterotetrameric receptor of 105 kDa (α) and 95 kDa (β) was found in the toad retina, which bound both insulin and insulin-like growth factor-1 (IGF-1) with equal affinity *(27).* Since these studies were published (latest in 1991), most of the research on insulin receptors in the retina has concentrated on their role in retinal development, usually in the chick retina *(28,29).* Very recently, the expression of two insulin receptors (*gfIR-1* and *gfIR-2*) has been reported in the goldfish retina, based on two partially isolated cDNAs as well as two transcripts (11 and 7 kb) on Northern blot analysis *(30).* A comparison between *gfIR-1* and *gfIR-2* showed that they are 69% identical *(30).*

Insulin in Photoreceptor Function

Insulin receptors are located on both the inner and outer segments of vertebrate photoreceptors *(31),* and insulin has been shown to produce a dose-dependent decrease in the a- and b-wave amplitudes of the electroretinogram (ERG), which is a measure of retinal function *(32).* The ERG b-wave reflects the activity of input from photoreceptors onto outer nuclear depolarizing bipolar cells *(32).* Suppression of the b-wave therefore suggests that insulin may affect transmitter release from photoreceptors. Transmission from photoreceptors is regulated by the activity of L-type Ca^{2+} channels (Ica) *(32).* It has been shown recently that insulin inhibits voltage-dependent calcium influx into rod photoreceptors, and this inhibition is mediated by the stimulation of insulin receptor tyrosine kinase activity *(32).* An insulin receptor-specific tyrosine kinase inhibitor, HNMPA-(AM)3, prevented insulin from reducing the depolarization-evoked Ca^{2+} increase in rods *(32).* These results suggested that insulin inhibits Ca^{2+} influx through voltage-dependent Ica in rod photoreceptors via tyrosine kinase activity *(32).* Inhibitory effects of insulin on rod Ica may be neuroprotective. Blockade of L-type Ca^{2+} channels in the rods of rd (retinal degeneration) mice reduced the amount of photoreceptor degeneration, suggesting that photoreceptor Ca^{2+} channels may play an important role in apoptosis *(33).* Also consistent with a potential neuroprotective role is the finding that insulin can prevent cultured retinal neurons from undergoing apoptosis during retinal development and diabetes *(34).*

Direct Interaction of Insulin Receptor With p85 Subunit of PI3K

Research over the past decade has revealed that receptor tyrosine kinases play a central role in signaling pathways initiated by growth fac-

tors *(35,36).* Several studies have shown that retinal outer rod segments (ROS) contain intrinsic tyrosine kinase(s) that can be activated by light *(37)* to phosphorylate at least 10 ROS proteins *(38,39).* The retina is an integral part of the central nervous system (CNS), and in the late 1980s, it was found to contain insulin receptors. However, little research has been done on these receptors since these early reports, probably because of the absence of an identified intracellular target. Recently, we have reported that the *p85* regulatory subunit of PI3K interacts directly with the insulin receptor in retinal ROS, which leads to activation of the PI3K enzyme *(40).* This interaction can be achieved either by light in vivo *(41)* or through tyrosine phosphorylation of the insulin receptor in vitro *(40).* The studies linking PI3K activation through phosphorylation of the insulin receptor in ROS now provide physiological relevance for the presence of these receptors in the retina. This article focuses mainly on our recently identified regulation of PI3K through the insulin receptor *(40,41).*

Phosphoinositide 3-Kinase

Multiple forms of PI3K exists in higher eukaryotes; the class Ia enzymes are responsible for the generation of D-3 phosphoinositides in response to growth factors *(42–44).* Class Ia enzymes are heterodimers of regulatory and catalytic subunits *(45).* The regulatory *p85* subunit contains a Src homology 3 (SH3) domain capable of binding to proline-rich sequences, a region of homology to the breakpoint cluster region (BCR) gene product, a *p110* binding domain, and two SH2 domains (N and C terminal). The regulatory subunit maintains the *p110* catalytic subunit in a low-activity state in quiescent cells, and mediates activation by direct interaction with phosphotyrosine residues of activated growth-factor receptors or adapter proteins *(45).* The activated PI3K converts the plasma membrane lipid phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2) to phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P_3). The termination of PI3K signaling by degradation of PI-3,4,5-P_3 can be mediated by at least two different types of phosphatases. The Src-homology 2 (SH2)-containing phosphatases (SHIP1 and SHIP2) dephosphorylate the 5-position of the inositol ring to produce PI-3,4-P_2. Although this dephosphorylation impairs some signaling downstream of PI3K, PI-3,4-P_2 can also mediate PI3K-dependent responses, and may mediate events that are independent of those stimulated by PI-3,4,5-P_3. Loss of SHIP2 causes a dramatic increase in insulin sensitivity, suggesting that this phosphatase critically regulates PI3K signaling downstream of insulin *(46).* In contrast, phosphatase PTEN dephosphorylates the 3-position of PI-3,4,5-P_3 to produce PI-4,5-P_2 *(47).* Loss of PTEN protein function has been found in a large fraction of a advanced cancers, indicating that uncontrolled signaling through PI3K may contribute to metastatic cancers *(48).* The PI3K pathway is implicated in human diseases such as diabetes and cancer, and an understanding of the complexities of this pathway may provide new avenues for therapeutic intervention.

Results and Discussion

Tyrosine Phosphorylation in the Regulation of PI3K

The involvement of protein tyrosine phosphorylation in the regulation of PI3K activity is well-documented *(49–53).* In response to various stimuli such as PDGF *(54)* and insulin *(55),* tyrosine phosphorylation of *p85* has been shown to occur in several cell types. In most cases, PI3K is regulated through receptor and non-receptor tyrosine kinases, without apparent phosphorylation of the *p85* subunit *(56–58).* Using in vitro conditions that favor tyrosine phosphorylation in ROS, we found ~2× greater PI3K activity from anti-*p85* and anti-*p110*α immunoprecipitates (IP) in PY-ROS (phosphorylated) than in N-ROS (non-phosphorylated) (Fig. 1), as previously shown *(59).* The increase of PI3K activity in anti-*p85* and anti-*p110*α IPs from PY-ROS could be caused by a greater amount of enzyme in the IP, activation of the enzyme by phosphorylation, or activation by some other mechanism. These pos-

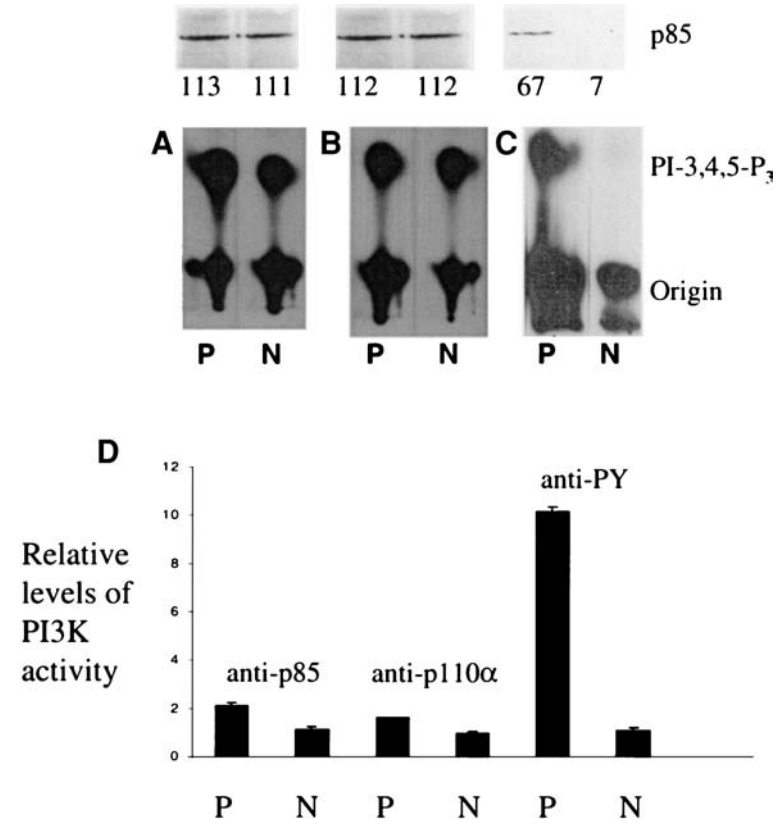

Fig. 1. TLC autoradiograms of PI3-kinase activity in **(A)** anti-*p85,* **(B)** anti-p110α, and **(C)** anti-PY immunoprecipitates of PY-ROS (P) or N-ROS (N), using PI-4,5-P_2 and [γ^{32}P]ATP as substrates (bottom panels). For Western blots (top panel), equal amounts of PY-ROS and N-ROS were immunoprecipitated either **(A)** anti-*p85,* **(B)** *p110*α, or **(C)** anti-PY antibodies and probed with anti-*p85* antibody. The values under each blot are the relative density of *p85* determined by scanning densitometry. **(D)** Relative levels of PI3K activity in anti-*p85,* anti-*p110,*α, and anti-PY IPs. The level of PI3K in N-ROS was set as I.0 for comparison. Values are mean and standard deviation for four independent ROS preparations. Reprinted with permission from *(40).*

sibilities were tested. When anti-*p85* and anti-*p110*α IPs from PY-ROS and N-ROS were subjected to Western blot analysis and probed with anti-*p85* or anti-*p110*α antibodies, the amount of *p85* and *p110*α were the same. Blots probed with anti-PY antibody showed that *p85* and *p110*α were not phosphorylated, suggesting that the twofold increase in enzyme activity was most likely caused by the influence of some tyrosine-phosphorylated protein in the IPs.

Anti-PY IPs from PY-ROS and N-ROS were assayed for PI3K activity and for the presence of *p85* protein. PI3K activity recovered from anti-PY IPs from PY-ROS was 10× that from N-ROS, and Western blots probed with the anti-*p85* antibody showed that the amount of *p85* in IPs from PY-ROS was about 10× more in the IPs from N-ROS. These results indicate that the *p85* regulatory subunit is most likely bound to, and co-immunoprecipitates with, a tyrosine phosphorylated protein, in response to phosphorylation of ROS.

Identification of a 97-kDa Protein Through Src Homology 2 (SH2) Domain of p85 Subunit of PI3K

Several studies have shown that the β-subunit of the insulin receptor and the IGF-1 receptor

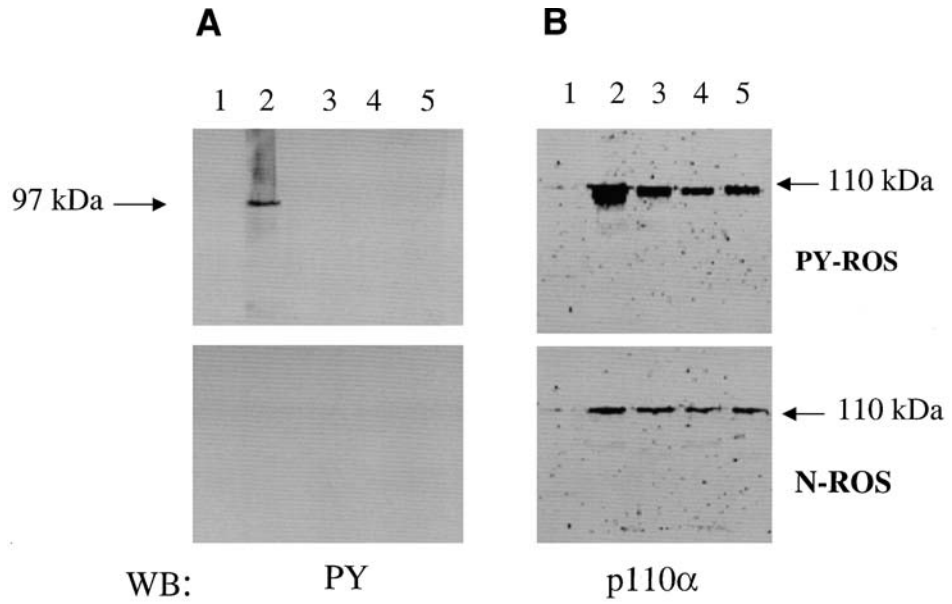

Fig. 2. GST pull-down experiments. PY-ROS and N-ROS were incubated with **(1)** GST, **(2)** GST-*p85* (N-SH2), **(3)** GST-*p85* (C-SH2), **(4)** GST-*p85* (N-SH2, R358A), or **(5)** GST-*p85* (C-SH2, R649A) fusion proteins, followed by Western blot analysis of the bound proteins with anti-PY antibody **(A)**. The blot was stripped and reprobed with anti-p110α antibody **(B)**. Reprinted with permission from *(40).*

can be phosphorylated in whole retina and ROS in response to insulin *(24,25)* and IGF-1 *(60,61).* Rodrigues et al. *(31)* used immunocytochemistry techniques to demonstrate that the insulin receptor is localized in photoreceptor and neuronal-cell bodies, with lower immunoreactivity in ROS. A 97-kDa protein in ROS that was actively phosphorylated in vitro under conditions that favor tyrosine phosphorylation was reported by Bell et al. *(38,39),* and Ghalayini et al. *(37)* showed that a 97-kDa protein was phosphorylated in rat ROS in a light-dependent manner in vivo. Based on these studies, we suspected that the 97-kDa protein we found bound to the *p85* regulatory subunit of PI3K could be the IRβ subunit, and the experiments reviewed here show that this is indeed the case. A variety of cytoplasmic proteins involved in mediating signals from cell-surface receptors to various intracellular pathways contain Src homology 2 (SH2) domains *(62).* They fold as molecular units, and are capable of recognizing and binding to proteins and linear peptide sequences containing phosphorylated tyrosine residues *(63).* We constructed two glutathione-*S*-transferase (GST)-*p85* (SH2) fusion proteins (N- and C-SH2 domains) and its respective mutants in an attempt to identify and characterize the tyrosine phosphorylated protein(s) in ROS. GST pull-down assays on PY-ROS and N-ROS followed by Western blot analysis employing an anti-PY antibody indicated the binding of a 97-kDa tyrosine phosphorylated protein to GST-*p85* (N-SH2) fusion protein (Fig. 2), but not in the GST-*p85* (N-SH2, R358A) mutant fusion protein, suggesting the specificity of phosphorylation-dependent binding. Van Horn et al. *(18)* reported a similar finding, although they also observed a low level of binding to the C-terminus of their GST-*p85* fusion protein. There was an increase in binding of p110α and an increase in PI3K activity in the presence of the 97-kDa protein bound to the GST-p85 (N-SH2) domain, which suggests that a *p85/p110*α/97-kDa protein complex may be sufficient to explain the increased PI3K activity in anti-PY IPs. The apparent mol wt of the phosphorylated protein bound by the GST-*p85* (N-SH2) fusion protein

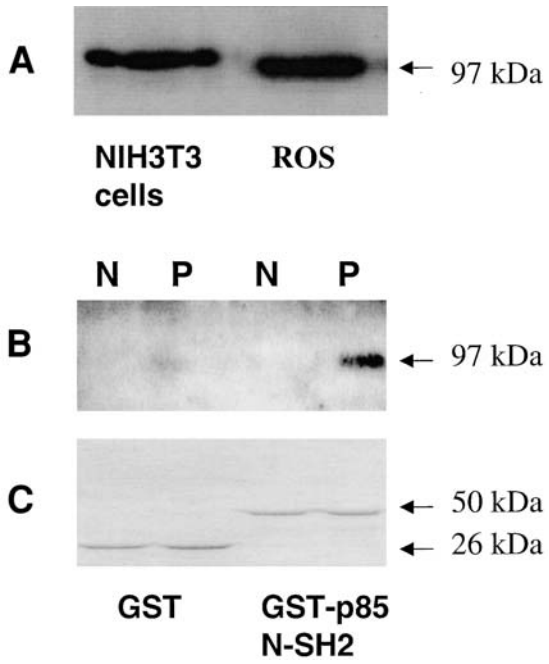

Fig. 3. Identification of the 97-kDa protein as the insulin receptor β-subunit. **(A)** 20 μg of ROS were subjected to immunoblot with anti-IRβ. Protein from NIH-3T3 cells transfected with the insulin receptor served as a positive control. **(B)** GST pull-down assays were carried out on PY-ROS and N-ROS incubated with GST and GST-*p85* (N-SH2) fusion proteins. These fusion proteins were washed and subjected to Western blot analysis with an anti-IRβ antibody. **(C)** The blot was stripped and probed with anti-GST antibody. Reprinted with permission from *(40).*

was similar to that of the β-subunit of insulin receptor *(24).* Western blots of ROS probed with an antibody to IRβ revealed an abundant presence of IRβ in these membranes. NIH-3T3 cells transfected with the insulin receptor served as a positive control. To test the possibility that the 97-kDa protein identified as IRβ is involved in PI3K binding, GST and GST-*p85* (N-SH2) fusion protein was incubated with PY-ROS or N-ROS, and each was subjected to GST pull-down assays, after which the fusion proteins were resolved by SDS-PAGE and the resultant Western blots were probed with anti-IRβ antibody. An immunoreactive band of IRβ was observed only in PY-ROS that were incubated with the GST-*p85* (N-SH2) domain (Fig. 3). The blot was then stripped and reprobed with an anti-GST antibody to demonstrate that equal amounts of GST were present in each sample (Fig. 3).

Effect of Insulin on PI3K

PI3K is known to be activated in other tissues by insulin *(64),* although there is no evidence that the insulin receptors in the retina undergo any physiological response following insulin stimulation. We observed a basal level of insulin-receptor phosphorylation in N-ROS accompanied by low PI3K activity (Fig. 4, lanes 2, 4, and 6), which increased significantly under tyrosine phosphorylation conditions (Fig. 4, lanes 1–2). This was also true when the ROS were incubated in the presence of insulin, followed by immunoprecipitation with anti-IRβ (Fig. 4, lanes 3–4). Increased PI3K activity was also observed from the IPs with anti-PY in insulin-treated ROS (Fig. 4, lanes 5–6), further confirming that insulin-induced tyrosine phosphorylation/autophosphorylation of IRβ leads to increased PI3K activity.

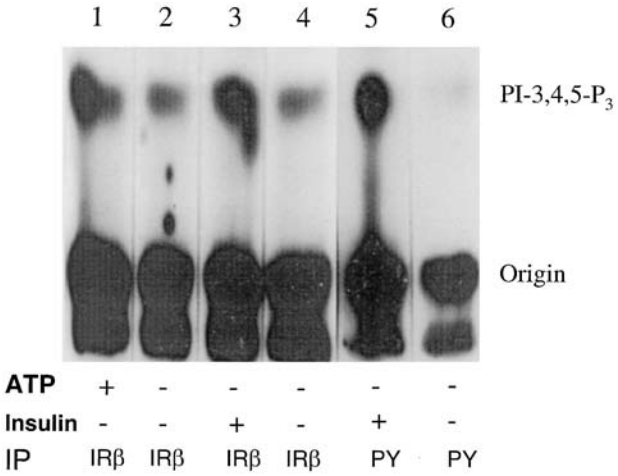

Fig. 4. TLC autoradiograms demonstrating the effects of phosphorylation and insulin on PI3K activity. ROS were either phosphorylated (lane 1) or non-phosphorylated (lane 2), or treated with (lane 3) or without (lane 4) insulin, and subjected to immunoprecipitation with anti-IRβ antibody. PI3K activity associated with anti-IRβ immunoprecipitates was measured. N-ROS were either incubated with (lane 5) or without (lane 6) insulin and immunoprecipitated with anti-PY antibody and measured for PI3K activity. Reprinted with permission from *(40).*

YXXM Motif of IRβ in the Regulation of PI3K Activity

Binding of the regulatory *p85* subunit of PI3K to phosphotyrosine at a YXXM motif *(65)* in IRS-1 activates the catalytic *p110* subunit *(14).* However, inactivation of the IRS-1 gene in the mouse using the homologous recombination approach did not result in any dramatic pathological phenotype, suggesting the possible existence of alternative signaling pathways *(66,67).* The wild-type insulin receptor binds tightly to the SH2 domains of *p85,* whereas the mutant insulin receptor truncated by 43 amino acids at the C-terminus binds weakly to the SH2 domains that lack the Y1322 THM motif *(18).* We cloned and expressed this sequence in bacteria under the control of an inducible promoter, to examine the binding properties of the non-catalytic regions of the insulin receptor containing the YTHM motif. When the tyrosine residue in the sequence becomes phosphorylated, it mimics receptor autophosphorylation normally induced by ligand binding. We found that the phosphorylated C-terminal tail of the insulin receptor bound to the *p85* subunit of PI3K in PY-ROS, and that the resulting complex contained PI3K activity (Fig. 5). These results suggests that non-catalytic cytoplasmic regions of growth-factor receptors provide phosphorylation-dependent binding sites for SH2-containing signaling proteins. Also, since there was no binding of IRS-1 to the phosphorylated C-terminal tail of the insulin receptor (data not shown), the evidence supports a direct interaction of *p85* subunit of PI3K with the cytoplasmic tail of the insulin receptor, independent of IRS-1.

These in vitro studies show that the insulin receptor is present in ROS, and binds directly to PI3K after phosphorylation of a tyrosine residue in its C-terminus. In the following sections, we describe our in vivo studies showing that light absorption by rhodopsin leads to phosphorylation of the insulin receptor and subsequent activation of PI3K.

Increased PI3K Activity In Vivo Associated With Anti-PY But Not With Anti-p85 IPs

Ghalayini et al. *(37)* previously reported that light stimulates tyrosine phosphorylation of multiple proteins in ROS in vivo. To determine whether light has an effect on PI3K activity and the phosphorylation of the insulin receptor, rats were dark-adapted overnight, and one-half were subjected to normal room light for 30 min. Retinal lysates were immunoprecipitated with anti-PY or anti-*p85* antibodies, and PI3K enzyme activity was higher in anti-PY IPs from whole retinas (Fig. 6A) and ROS (Fig. 6B) from light-adapted (L) rats, compared to those from dark-adapted (D) animals. A marginal increase in PI3K activity was observed in anti-*p85* IPs of light-adapted retinas over dark-adapted retinas (Fig. 6C), suggesting that light may induce the translocation of PI3K from cytoplasm to tyrosine-phosphorylated proteins in the disk membrane.

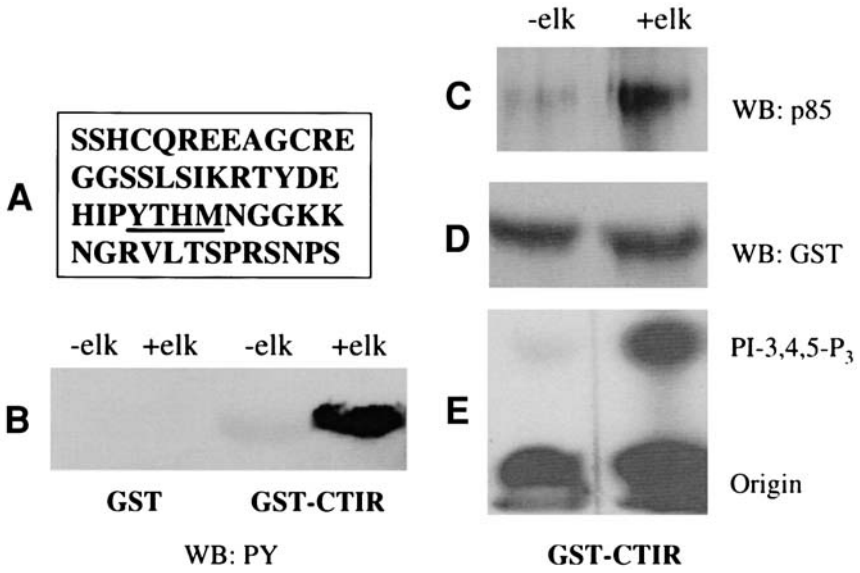

Fig. 5. Effect of phosphorylation of the insulin receptor on PI3K activity. **(A)** C-terminal tail of insulin receptor (CTIR) cloned from rat retina by reverse transcription polymerase chain reaction. The *YTHM* motif is underlined. Co-expression of GST/GST-CTIR along with or without *elk* tyrosine kinase, and the expressed proteins were immunoblotted with anti-PY **(B)**, incubated with non-phosphorylated ROS followed by GST pull-down assays and immunoblotted with anti-*p85* **(C)**. PI3K activity associated with either non-phosphorylated or phosphorylated CTIR were measured using PI-4,5-P_2 as substrate **(E)**. The blot was stripped and probed with anti-GST antibody **(D)**. Reprinted with permission from *(40).*

*Increased IR*β *Phosphorylation, p85 Association, and PI3K Enzyme Activity Associated With Anti-IR*β *IPs of Light-Adapted Rat Retinas*

Western blot analysis of anti-IRβ IPs probed with anti-IRβ antibody indicated an equal amount of IRβ in both light- and dark-adapted rat retinas (Fig. 7A). However, probing with anti-PY (Fig. 7B) and anti-p85 (Fig. 7C) antibodies showed almost twofold greater phosphorylation of IRβ in light-adapted rat retinas and binding of *p85*, respectively. In these anti-IRβ IPs, PI3K activity was more than fourfold higher in light-adapted rats compared to those from dark-adapted animals (Fig. 7D, E), suggesting that light induced phosphorylation of the IRβ, as well as binding of *p85* to the insulin receptor.

Light-Dependent Association of p85 With ROS Membranes

The studies described here were done on whole retinal homogenates. To determine whether the light effect occurred in the photoreceptor outer segment, purified ROS membranes were prepared on a discontinuous sucrose density gradient. Greater PI3K enzyme activity was found in ROS membranes from light-adapted rats compared to dark-adapted rats (Fig. 8D), and Western blot analysis indicated an increased amount of *p85* protein in light-adapted ROS compared to dark-adapted ROS (Fig. 8A). The photoreceptor specific protein opsin was used as internal control and indicated that an equal amount of protein was used in these analyses (Fig. 8C). Another control was arrestin, which is known to move from

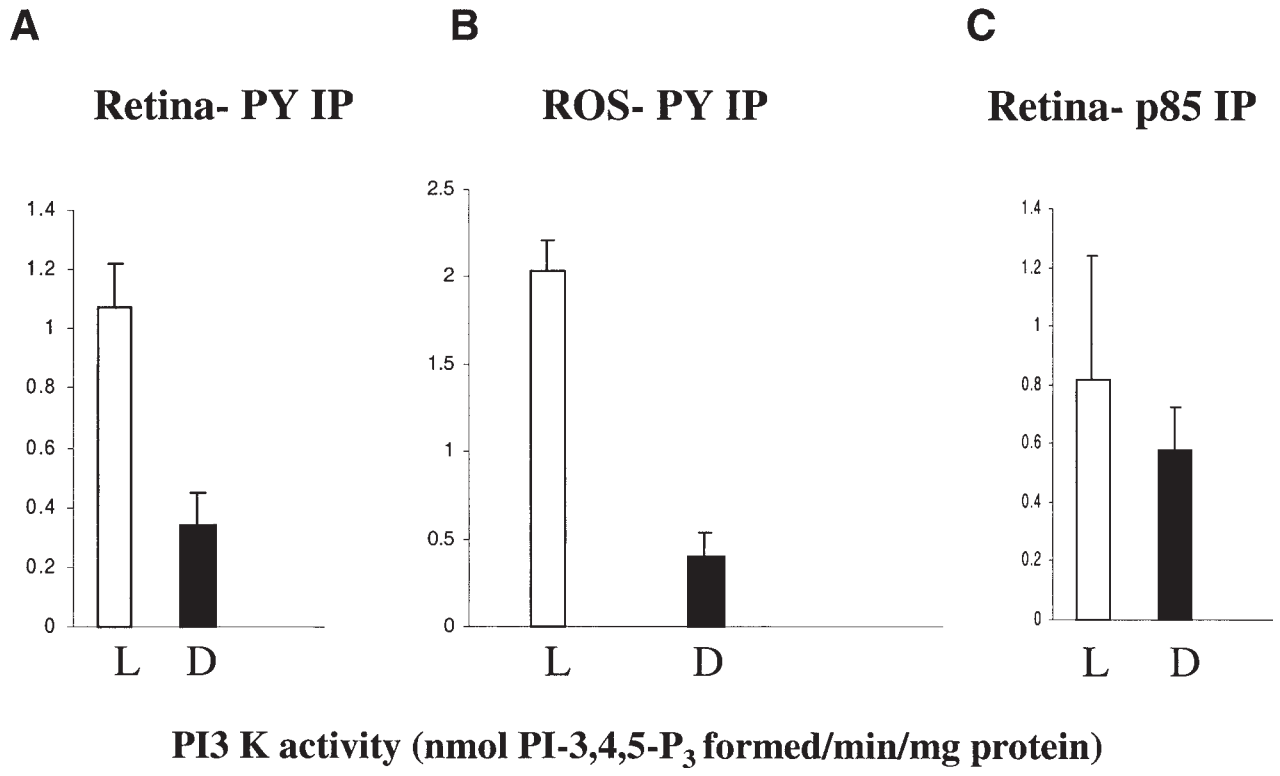

Fig. 6. PI3K enzyme activity in anti-PY **(A)** or anti-p85 **(C)** immunoprecipitates from dark- or light-adapted rat retina homogenates, or ROS immunoprecipitated with anti-PY **(B)**. PI3K activity was measured using PI-4,5-P_2 and [γ^{32}P]ATP as substrates. The radioactive spots of PI-3,4,5-P_3 were scraped from the TLC plate and counted. Reprinted with permission from *(41).*

the inner segment to the outer segment in light *(68,69).* Its accumulation in the ROS verified that the retinas responded to light in a predictable manner (Fig. 8B). The in vivo studies presented in Figs. 7 and 8 demonstrate the light-induced tyrosine phosphorylation of IRβ and the subsequent association of PI3K in ROS.

Role of Endogenous Insulin in the Activation of the Retinal Insulin Receptor

The role of endogenous insulin in retinal IRβ phosphorylation was determined by measuring IRβ activation in rats that were made insulin-deficient by the injection of streptozotocin. The state of diabetes was confirmed by measuring blood glucose levels *(70).* Retinas from hyperglycemic (insulin-deficient animals) and control animals in normal room light were lysed in homogenizing buffer immunoprecipitated with anti-IRβ antibody. No difference in the PI3K activity (Fig. 9B) or the degree of IRβ phosphorylation (Fig. 9A) was found between and insulin-deficient rats. These studies suggest that although insulin may be sufficient to activate insulin receptors in isolated ROS in vitro *(24,25,40),* it may not be required for the light-dependent activation of IRβ in vivo.

Role of Photobleachable Visual Pigments in the Activation of PI3K

We examined wild-type and Pdeb(rd) mutant mice (FVB), which lack photoreceptors, to determine the involvement of photobleachable visual pigments in the regulation of PI3K activity. As previously shown in rat retinas (Fig. 6–8), PI3K activity was significantly higher in

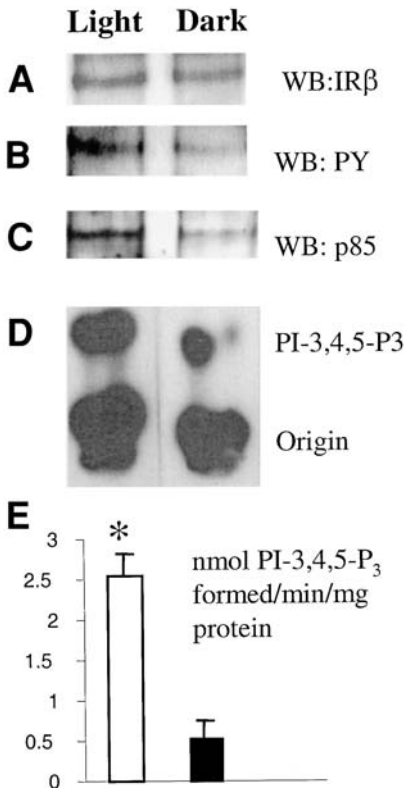

Fig. 7. In vivo light-dependent phosphorylation of IRβ. Rats were either dark- or light-adapted, and retinas from each rat were pooled, homogenized, immunoprecipitated with anti-IRβ antibodies, and immunoblotted with anti-IRβ **(A)** or anti-PY **(B)**, anti-*p85* antibodies **(C)** or measured for PI3K activity using PI-4,5-P_2 and [γ^{32}P]ATP as substrates **(D)**. The radioactive spots of PI-3,4,5-P_3 from the TLC plate were scraped and counted **(E)**. Reprinted with permission from *(41).*

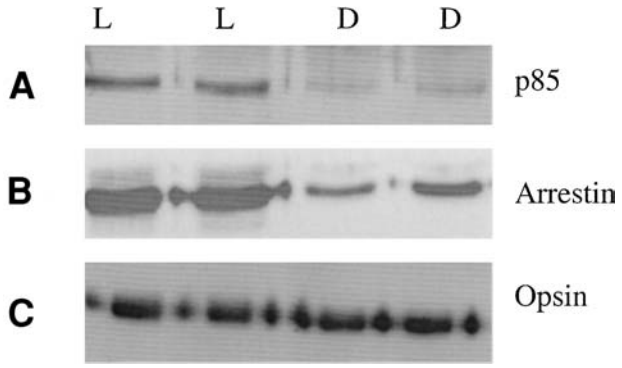

Fig. 8. PI3K activity and expression of *p85* **(A),** arrestin **(B),** and opsin **(C)** in dark- and light-adapted rat ROS. Reprinted with permission from *(41).*

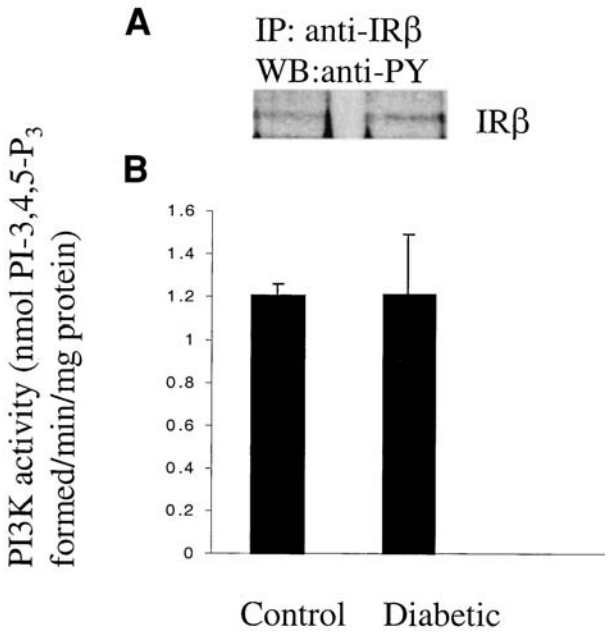

Fig. 9. PI3K activity and phosphorylation of IRβ in control and diabetic rat retinas. Retinal protein was immunoprecipitated with anti-IRβ antibody and subjected to either Western blotting analysis with anti-PY antibody **(A)** or measuring PI3K activity **(B)**. Reprinted with permission from *(41).*

anti-IRβ IPs of light-adapted wild-type mouse retinas compared to dark-adapted mouse retinas (Fig. 10A). However, there was no difference in PI3K activity in light- and dark-adapted mutant mice (Fig. 10B). The absence of photoreceptors was confirmed by probing the retina lysates with an anti-opsin antibody, in which no detectable opsin was observed (Fig. 10D) compared to wild-type mouse retina (Fig. 10C); however, probing the FVB mouse retinas with anti-IRβ antibodies indicated the presence of IRβ (Fig. 10E), demonstrating that IRβ is also present in retinal cells other than photoreceptors. The results suggest that the observed light/dark differences in IRβ phosphorylation and subsequent binding of PI3K are a photore-

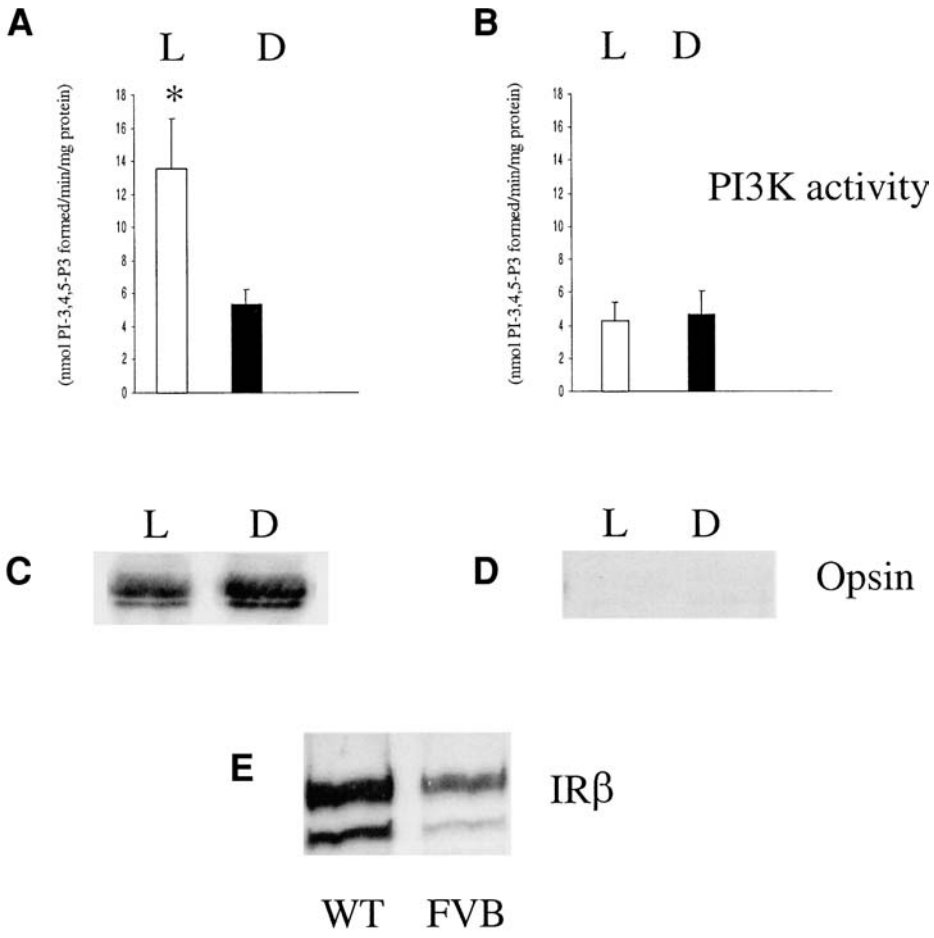

Fig 10. PI3K activity, opsin, and IRβ expression in retinas of wild-type and FVB mice. PI3K activity was measured in the immunoprecipitates of IRβ from **(A)** wild-type **(B)** FVB mouse retinas. Opsin expression was examined from light- and dark-adapted wild-type **(C)** and FVB mice **(D)**. IRβ **(E)** expression was examined only from light-adapted wild-type and FVB mice. D-dark, L-light. Reprinted with permission from *(41)*.

ceptor-specific phenomenon that are probably mediated by photon capture by rhodopsin in the ROS. This result excludes the possibility that the light-dependent activation occurs in photopigment-expressing ganglion cells, inner retinal neurons, or retinal pigment epithelium (RPE) *(71,72)*, since these cells are viable in the FVB mice.

*Mechanism(s) of Light-Stimulated Phosphorylation of IR*β

We have theorized that there could be at least two possible mechanisms that trigger the phosphorylation of IRβ. The first could involve ligand(s) other than insulin, which are induced or released in response to light and bind to IRβ. Candidates include activated transducin, or another participant in the visual transduction cascade. The second mechanism involves the activation of a non-receptor tyrosine kinase(s) in response to light. Non-receptor tyrosine kinase Src phosphorylates insulin- and IGF receptors on autophosphorylation sites, and Src kinase has been shown to substitute for the ligand-dependent receptor activation *(73,74)*. c-Src was recently shown to associate with light-activated opsin *(75)*.

Together, these results suggest a model in which the light-activation of opsin results in its association with c-Src, which in turn associates with and activates the insulin receptor. Consistent with this mechanism, we have also reported previously the in vitro phosphorylation of IRβ by c-Src in ROS *(40).*

Cross-Communication Between Signal Transduction and Phototransduction

The precise mechanism of light-dependent activation of IRβ and PI3K in photoreceptor cells is unknown. In other cell types, activation of the insulin-receptor pathway has been shown to have diverse physiological roles *(76).* The light-dependent IRβ activation in rod photoreceptor cells suggests that its function could be related to rod-specific activities that are governed by light. These functions include light adaptation *(77),* biogenesis of new ROS membranes through the addition of newly synthesized ROS membranes at the base of ROS *(78)* or shedding of photoreceptor tips *(79).* PI3K is a neuroprotective agent as has been demonstrated in cerebellar granular neurons *(80)* and PC12 cells *(81)* in which receptor activation of PI3K has been shown to protect these cells from stress-induced neurodegeneration. Bright light has been shown to cause the death of rod and cone photoreceptor cells *(82,83),* activation of PI3K at physiological lighting conditions could offer an innate self-protection mechanism. Consistent with this hypothesis, we have recently observed that PI3K downstream target cAkt was also found to be phosphorylated in response to light (unpublished observations), suggesting that light-induced PI3K activation through IRβ could modulate the functions of downstream signaling intermediates. Homozygous Pdeb-mutant mice that lack rods and cones failed to activate PI3K in a light-dependent manner, suggesting that proteins, which are involved in the phototransduction cascade, could regulate the activation of IRβ and subsequent regulation of PI3K activity. This observation raises several intriguing questions: Which part of the visual cycle is sufficient in this activation step, such as bleaching of rhodopsin? What is the role of G-proteins? Is there an involvement of channels? Transgenic mouse models carrying various mutant proteins of the visual transduction pathway would allow dissection of the molecular mechanism (s) of light-induced activation of IRβ. Studies are currently underway in our laboratory to address these objectives.

Acknowledgments

This work was supported by grants from the National Institutes of Health (EY00871, EY04149, EY12190, and RR17703), Research to Prevent Blindness Inc., New York, NY; The Foundation Fighting Blindness, Baltimore, MD; Samuel Roberts Nobel Foundation, Inc., Ardmore, OK.

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