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CYTOSKELETAL COMPONENTS ENHANCES THE AUTOPHOSPHORYLATION OF RETINAL INSULIN RECEPTOR

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Abstract

Insulin Receptor (IR) signaling provides a trophic signal for transformed retinal neurons in culture, and we recently reported that deletion of IR from rod photoreceptors resulted in stress-induced photoreceptor degeneration. Retinal insulin receptor has a high basal level autophosphorylation compared to liver and the reasons for higher autophosphorylation are not known. In the current study we report a novel finding that cytoplasmic actin associates with and activates the retinal IR *in vivo*. Similar to insulin, actin also induced autophosphorylation at tyrosines 1158, 1162 and 1163 in the catalytic loop of IR. Our studies also suggest that globular actin activates the retinal IR more effectively than does filamentous actin. Retinal IR kinase activity has been shown to decrease in hyperglycemia and we found a decreased binding of actin to the IR under hyperglycemia. This is the first study which demonstrates that cytoplasmic actin regulates autophosphorylation of the retinal IR.

Keywords

Insulin receptor; Autophosphorylation; Actin cytoskeleton; Diabetes; Streptozotocin; Rhodopsin

1. Introduction

The actions of insulin are initiated by its binding to the IR, a disulfide-bonded heterotetrameric membrane protein [1–3]. Insulin binds to two asymmetric sites on the extracellular α subunits, causing conformational changes that lead to autophosphorylation of the membrane-spanning β subunits and activation of the receptor's intrinsic tyrosine kinase [4;5]. The IR transphosphorylates several immediate substrates on tyrosine residues, including IR substrate (IRS) proteins [6]. These events lead to the activation of downstream signaling molecules. Retinal cells contain specific high-affinity receptors for insulin [7]. We have shown previously

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Conflict of interest

None

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that light causes increased tyrosine phosphorylation of the retinal IR and this activation leads to the activation of phosphoinositide 3-kinase (PI3K) *in vivo* [8]. The light effect is localized to the photoreceptor neurons [8]. Very recently we have demonstrated that photobleaching of rhodopsin regulates the tyrosine phosphorylation of the retina IR [9]. IR signaling provides a trophic signal for transformed retinal neurons in culture [10], and we recently reported that deletion of IR from rod photoreceptors resulted in stress-induced photoreceptor degeneration [11]. Further, it has been reported that the retinal IR has high basal constitutive activity which is independent of circulating insulin [12;13]. A significant decrease of retinal IR kinase activity has been reported after 4 weeks of hyperglycemia in STZ treated rats [14]; however, the mechanism of this constitutive IR activation remains unknown. The high basal IR kinase activity [12] and light-induced activation of the IR [8] led to the hypothesis that novel ligands or regulators of the IR exist in the retina. Consistent with this hypothesis that ligand-independent activation of IR has been reported previously [15–18]. Two compounds capable of stimulating IR autophosphorylation by acting on the cytoplasmic domain have been reported [15;16]. One of these (L783,281) modestly elevates IR autophosphorylation in the absence of insulin [19], whereas the other (TLK16988) potentiates receptor autophosphorylation in the presence of insulin, suggesting two different mechanisms of action [18]. Structural and biochemical evidence for an autoinhibitory role for tyrosine 984 in the IR has been reported [20]. Substitution of tyrosine 984 in the β sheet- α C cleft with alanine resulted in increased basal level of IR phosphorylation [20]. Based on these results, it has been proposed that compounds that bind in the β sheet- α C cleft and displace tyrosine 984 should partially activate the IR [20]. Furthermore, regulation of IR kinase activity by phosphatidylinositol in the absence of insulin has also been reported [17]. These studies suggest that the cytoplasmic domain of the IR can be autophosphorylated independent of insulin.

In this study we report a novel finding that actin endogenously associates with and activates the retinal IR *in vivo*. Similar to insulin, actin induced the autophosphorylation of tyrosines 1158, 1162 and 1163 in the catalytic loop of IR. Our studies also suggest that G-actin more effectively activates the retinal IR than F-actin. Our studies demonstrate for the first time that actin may be one of the physiological regulators of the retinal IR.

2. Materials and methods

2.1. Materials

Polyclonal anti-IR β and monoclonal anti-PY-99 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-insulin/IGF-1-like growth factor-1 receptor (pYpYpY^{1158, 1162, 1163}) phosphospecific (pIR) antibody was obtained from Biosource (Camarillo, CA). Anti-His antibody was obtained from Cell Signaling (Beverly, MA). [γ ³²P] ATP was obtained from New England Nuclear (Boston, MA). Actin antibody was obtained from Affinity BioReagents (Golden, CO). Human recombinant β -insulin receptor kinase-GST fusion was obtained from Calbiochem (San Diego, CA). Skeletal muscle actin was obtained from Sigma (St Louis, MO). TNT-T7 quick coupled transcription/translation reagents were obtained from Promega (Madison, WI). All other reagents were of analytical grade and from Sigma.

2.2. Animals

All animal work was in strict accordance with the *NIH Guide for the Care and use of Laboratory Animals* and the Association for Research in Vision and Ophthalmology on the Use of Animals in Vision Research. All protocols were approved by the IACUC at the University of Oklahoma Health Sciences Center and the Dean McGee Eye Institute. C57BL/6 mice (4–6 weeks old, male) were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in our vivarium in cyclic light (12h on; 12 h off; ~ 300 lux). Mice were acclimated with a 12-hour

light/dark schedule and fed standard laboratory food and water *ad libitum* until the induction of hyperglycemia.

2.3. Generation of hyperglycemic mice

Hyperglycemia was induced by a series of two injections. At eight weeks, mice were weighed and given an intraperitoneal injection (100mg/kg) of streptozotocin (STZ) in freshly dissolved citrate buffer (10 mmol, pH 4.5). Control animals were given an intraperitoneal injection of citrate buffer only. At twelve weeks, mice were weighed and blood glucose levels were analyzed. Mice with blood glucose levels >250 mg/dl (TrueTrack Smart System; AR-MED Ltd, Egham, UK) were considered hyperglycemic. Mice were euthanized by CO₂ asphyxiation and cervical dislocation. Retinas were immediately removed after euthanization and frozen in liquid nitrogen.

2.4. Retinal tissue preparation

Either rats or mice were killed by CO₂ asphyxiation followed by cervical dislocation. Retinas were quickly removed by a technique called “winkling” [21]. A deep cut is made across the corneal surface and curved forceps are placed behind the eyeball on either side of the optic nerve head. The forceps are squeezed and brought forward, forcing the contents of the eye to be extruded through the hole in the cornea. The retina can be recovered relatively intact and placed immediately into ice-cold buffer or snap frozen with liquid nitrogen. Retinas were homogenized by hand in lysis buffer [1% NP 40, 20 mM HEPES (pH 7.4), 2 mM EDTA, phosphatase inhibitors (100 mM NaF, 10 mM Na₄P₂O₇, 1 mM NaVO₃, and 1 mM molybdate), and protease inhibitors (10 μM leupeptin, 10 μg/ml aprotinin and 1 mM PMSF)]. The lysates were kept on ice for 10 min followed by centrifugation at 4 °C for 20 min.

2.5. Insulin receptor autophosphorylation

Retinal lysates were immunoprecipitated with anti-IRβ antibody. Analysis of IR autophosphorylation was carried out as previously described [17]. Briefly, IR immunoprecipitates were incubated either in the presence or absence of 1 μM human insulin, assay buffer [HEPES (pH 7.4), 10 mM MnCl₂, 10 mM MgCl₂, and 0.65% n-octyl-β-D-glucopyranoside] and ATP to a final concentration of 100 μM. The reaction was incubated at room temperature for 60 min. Sodium phosphate buffer has been shown to stabilize the phosphotyrosine in proteins compared to that of sample buffer containing Tris [22;23]. Therefore, sodium phosphate sample buffer [0.062 M phosphate, pH 7.0, 10% w/v glycerol, 2% SDS, 0.001% bromophenol blue and 5% 2-mercaptoethanol] was added to the immunoprecipitates and the suspension boiled at 100 °C for 5 min. The samples were run on 10% SDS-PAGE followed by Western blot analysis with either anti-pIR or anti-phosphotyrosine (PY-99) antibodies.

2.6. Peptide mass fingerprinting (PMF)

The IR was immunoprecipitated with anti-IRβ antibody from retinal lysates and the bound proteins were resolved on SDS-PAGE followed by Gel Code blue staining. The visualized 42 kDa protein on the gel was subjected to in-gel digestion as previously described [24;25]. Mass spectra were obtained using a MALDI-TOF MS (Voyager Elite, Applied Biosystems, Foster City, CA). The PMF search was performed by MASCOT (<http://www.matrixscience.com>) using the NCBI nr database.

2.7. Cloning of beta actin from mouse retina

Retinal actin was obtained by PCR of reverse transcribed retinal RNA using 5' and 3' oligonucleotides, designed based on mouse actin (sense: AAG CTT CAT CAT CAT CAT CAT CAT ATG GAT GAC GAT ATC GCT GCG CTG; antisense: GAA TTC CTA GAA

GCA CTT GCG GTG CAC G). We added a histidine tag to the N-terminal end of the actin. The cDNA encoding full-length mouse actin was cloned into a TOPO vector and sequenced. The cDNA insert was excised from the TOPO vector as a HindIII/EcoRI fragment and cloned into a pCDNA3 mammalian expression vector.

2.8. Site-directed mutagenesis of actin

Site-directed mutagenesis (SDM) was carried out with the Quickchange site-directed mutagenesis kit (Stratagene Inc, LaJolla, CA) using a PTC 200 programmable thermal controller (MJ Research, Inc., Watertown, MA). The reaction mixture contained SDM buffer [200 mM Tris-HCl (pH 8.8), 100 mM KCl, 100 mM (NH₄)₂SO₄, 20 mM MgSO₄, 1% Triton X-100, 1 mg/ml nuclease-free bovine serum albumin, 1 mM deoxynucleotide mix (dATP, dCTP, dTTP and dGTP), 50 ng of vector and 125 ng of sense and antisense primers with mutations] in a total volume of 50 µl, followed by the addition of 2.5 units of pfu DNA polymerase. The primers used in the SDM are as follows: S14C (sense: GTC GAC AAC GGC TGC GGC ATG TGC AAA GCC; anti-sense: GGC TTT GCA CAT GCC GCA GCC GTT GTC GAC) and R62D (sense: GAG GCC CAG AGC AAG GAC GGT ATC CTG ACC; anti-sense: GGT CAG GAT ACC GTC CTT GCT GGC CTC). The extension parameters of SDM were as follows: initial denaturation at 95 °C for 30 sec, followed by 16 cycles at 95 °C for 30 sec, 55 °C for 1 min and at 68 °C for 12 min (2 min/kb of plasmid length). Following temperature cycling, the reaction was placed on ice for 2 min, after which 10 units of DPN 1 restriction enzyme were added, mixed and incubated at 37 °C for 60 min. Transformation was carried out by adding 1 µl of the DPN 1 treated reaction mixture to Epicurean XL-blue super-competent cells at 4 °C for 30 min, followed by 60 min at 37 °C with shaking. The reaction mixture was then placed on LB/Amp (100 µg/ml) plates. The cDNAs of all mutants were then sequenced after PCR, and the only mutations observed were those intentionally introduced to create specific mutations. After sequencing, actin mutants were excised from TOPO vector as HindIII/EcoRI and cloned into pCDNA3 vector. Plasmid DNAs of actin mutants were prepared and used in *in vitro* coupled transcription and translation.

2.9. *In vitro* coupled transcription and translation

TNT-T7 quick coupled transcription/translation system (Promega, Madison, WI) was used for the synthesis of wild-type, S14C and R62D mutant actins. The reaction mixture contained 40 µl of TNT quick master mix, 1 µl of 1 mM methionine and 1 µg cDNA (either wild-type or S14C or R62D actin mutants) in a final volume of 50 µl. The reaction was incubated at 30 °C for 90 min. Protein expression was examined by Western blot analysis employing anti-His antibody.

2.10. Rhodopsin measurement

Eighty-day-old citrate buffered control and STZ (6 weeks after injection) mice were dark-adapted overnight, sacrificed in the morning, and the eyes were removed under dim red light. A single eye from each mouse was homogenized in 100 µl of 1 X PBS [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, and 1.4 mM KH₂PO₄] containing 2% *N*-octyl-β-D-glucopyranoside (Calbiochem, La Jolla, CA) and 50 mM of freshly prepared hydroxylamine. The suspension was centrifuged at 16,100 × g for 5 min and the supernatant was collected into a fresh eppendorf tube. The volume was measured for each sample and the clear supernatant was scanned from 200 to 800 nm (Ultrospec 3000; Amersham Biosciences, Cambridge, UK), after which the samples were bleached by exposure to room light for at least 5 min and scanned again. The difference in absorption at 500 nm was used to determine the concentration of rhodopsin using a molar extinction coefficient of 42,000 [26]. The concentration of rhodopsin was expressed as pmol/eye.

2.11. Immunoprecipitation

Retinal lysates were pre-cleared by incubation with 40 μ l of protein A-Sepharose for 1 h at 4 °C with mixing. The supernatant was incubated with anti-IR β (4 μ g) antibody overnight at 4 °C and subsequently with 40 μ l of protein A-Sepharose for 2 h at 4 °C. Following centrifugation at 14,000 rpm for 1 min, immune complexes were washed three times with wash buffer [50 mM HEPES (pH 7.4) containing 118 mM NaCl, 100 mM NaF, 2mM Na₃VO₃, 0.1% (w/v) SDS and 1% (v/v) Triton X-100]. Immunoprecipitates were subjected to immunoblot analysis with anti-PY99 (1:1000), anti-IR β (1:1000), anti-pIR (1:1000), or anti-actin (1:1000) antibodies.

2.12. SDS-PAGE and Western blotting

Proteins were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The blots were washed twice for 10 min with TTBS [20 mM Tris-HCl (pH 7.4), 100 mM NaCl and 0.1% Tween-20] and blocked with either 5% bovine serum albumin or non-fat dry milk powder (Bio-Rad) in TTBS for 1 h at room temperature. Blots were then incubated with anti-IR β (1:1000), anti-PY99 (1:1000), anti-actin (1:1000), anti-His (1:1000) or anti-IR-pYpYpY1158, 1162, 1163 (1:1000) antibodies overnight at 4 °C. Following primary antibody incubations, immunoblots were incubated with HRP-linked secondary antibodies (either anti-rabbit or anti-mouse) and developed by ECL according to the manufacturer's instructions. Densitometric analyses of immunoblots were performed using a Kodak Image Station 4000R (Eastman Kodak Company, Rochester, NY) in the linear range of detection. Absolute values were then normalized to total protein as indicated in the figure legends.

2.13. Statistical methods

Results are presented as mean and standard deviation. One-way ANOVA with unpaired t-tests were used for assessing significant differences across groups for the effect of actin on IR kinase activity. Probability values <0.05 are reported as significant.

2.14. Other methods

G-actin was polymerized into F-actin by incubation with 100 mM KCl at room temperature for 2 h as reported [27]. Bovine rod outer segments (ROS) were prepared according to the method described earlier [8].

3. Results

3.1. Increased autophosphorylation of retinal IR is not a contamination of non-receptor tyrosine kinases

It has been shown that the non-receptor tyrosine kinase Src phosphorylates insulin and insulin-like growth factor receptors on autophosphorylation sites (45; 46). Thus, the Src kinase has been shown to substitute for ligand-dependent receptor activation (44; 45). To determine whether the previously reported autophosphorylation of the retinal IR [28] is not due to the contamination of non-receptor tyrosine kinase(s), we have carried out the autophosphorylation assay on anti-IR β immunoprecipitates (Fig. 1B) in the presence of non-receptor tyrosine kinase inhibitors PP1, PP2, and PP3 (a negative control for the Src family protein tyrosine kinase inhibitor PP2). PP1 and PP2 failed to inhibit the autophosphorylation of IR (Fig. 1A), suggesting that the observed IR autophosphorylation is not mediated through non-receptor tyrosine kinase(s).

To confirm the effect of PP1 and PP2 on the inhibition of non-receptor tyrosine kinases, we carried out the *in vitro* phosphorylation of bovine ROS in the presence and absence of PP1, PP2, and PP3. This was followed by Western blot analysis with anti-PY99 antibody. The results

indicate that PP1 and PP2 completely block the phosphorylation of ROS proteins (Fig. 1C), suggesting the inhibition of non-receptor tyrosine kinase activity by PP1 and PP2. To ensure equal amounts of protein in each reaction, we reprobed the blot with arrestin, a photoreceptor specific protein (Fig. 1D). These experiments further attest that the IR autophosphorylation is not due to the involvement of non-receptor tyrosine kinase(s).

3.2. Identification of 42 kDa protein associated with retinal IR

To test the hypothesis that the IR associates with regulatory protein(s), IR was immunoprecipitated from retinal lysates with anti-IR β antibody and bound proteins were resolved by SDS-PAGE and stained with GelCode blue. A 42 kDa protein associated with the retinal IR was observed on stained gels (Fig. 2). The 42 kDa band was excised from the gel, digested with trypsin, and the peptide fragments were subjected to peptide mass fingerprinting. The obtained peptide masses were then subjected to a NCBIrr to identify any peptide/polypeptide that corresponded to the obtained mass. Four peptide fragments identified the 42 kDa protein as the cytoplasmic actin (Table 1).

3.3. Actin associates with the retinal IR in vivo

The association of IR and actin in the retina was studied using co-immunoprecipitation method. Lysates from retina were immunoprecipitated with antibody directed against IR or non-immune IgG. Immunoprecipitation of IR by anti-IR antibody resulted in the co-precipitation of actin (Fig. 3). The non-immune IgG failed to co-precipitate both IR and actin. These results suggest that IR is associated with actin in the retina.

3.4. Actin and IR interaction in retina and liver tissues

We [28] and others [14] have reported previously that retina has a high basal autophosphorylation compared to liver. To determine whether the observed differences are due to the actin association with IRs, we have subjected varying amounts (2.5 to 10 μ g) of retinal and liver proteins to Western blotting analysis with anti-IR and anti-actin antibodies. The results indicate IR (Fig. 4A) and actin (Fig. 4B) immunoreactivity was higher in retina compared to liver and this discrepancy is due to higher total protein in liver compared to retina. To determine the absolute levels of IR in retina and liver, we normalized the IR to actin and the results indicate that there was no difference in the levels of IR between retina and liver (Fig. 4C).

It has been shown previously that basal IR kinase activity in the retina was significantly greater than that of liver, and remained constant between freely fed and fasted rats suggesting, that IR activation was not regulated by circulating insulin [12;13]. In the retina, constitutive IR autophosphorylation may be a result of alternative splicing that skips exon 11 to produce a type A receptor [3;29;30]. To determine whether there is any difference in the actin binding to retinal IR compared to liver IR, we immunoprecipitated the IR from retina and liver tissues with anti-IR β antibody followed by Western blot analysis with the anti-actin antibody (Fig. 4D). The blot was reported with anti-IR antibody to ensure equal amounts of protein in each immunoprecipitate (Fig. 4E). We normalized the actin to IR and the results indicate a significantly increased amount of actin associated with the retinal IR compared to liver IR (Fig. 4F). This experiment suggests that actin has more affinity for retinal IR than liver IR.

To further determine the actin-induced autophosphorylation of IR, we immunoprecipitated IR from retinal and liver tissues (normalized IR) with anti-IR antibody (Fig. 4H) and carried out the autophosphorylation of IR in the presence of either 4.6 or 9.3 μ M actin. The results indicate a concentration dependent retinal IR activation compared to liver (Fig. 4G). This experiment further suggests that retinal IR kinase activity could be modulated by actin.

3.5. Actin-induced autophosphorylation is similar to insulin

The catalytic loops within the tyrosine kinase domain of the IR contain three (Y1158, Y1162 and Y1163) tyrosine motif [2;3]. It is generally believed that autophosphorylation within the activation loop proceeds in a processive manner initiated at the second tyrosine (1162), followed by phosphorylation at the first tyrosine (1158) and then the last (1163), upon which the IR becomes fully active [2;3]. To determine whether actin activates IR in the same manner as insulin, we immunoprecipitated the IR (Fig. 5C) from retinal lysates and then subjected the immune complexes to *in vitro* autophosphorylation in the presence of either insulin or actin (Fig. 4B) for 60 min at room temperature. At the end of incubation, SDS sample buffer was added followed by Western blot analysis with phosphospecific anti-pIR antibody. The results indicate that actin and insulin-induced autophosphorylation of IR are recognized by the phosphospecific IR antibody, suggesting that actin induces the autophosphorylation of IR in the catalytic loop similar to insulin (Fig. 5A). These results further suggest that there could be two pools of IR with bound and unbound actin that were recovered from IR immunoprecipitates. The IR activation by the addition of actin could be due to the binding of actin to the unbound IR.

To further confirm the dose dependent IR catalytic loop autophosphorylation by actin, we immunoprecipitated the IR (Fig. 5E) from retinal lysates and then subjected them to *in vitro* autophosphorylation in the presence of varying concentrations of actin for 60 min at room temperature. At the end of incubation, SDS sample buffer was added and the immune complexes were subjected to Western blot analysis with phosphospecific anti-pIR antibody. The results indicate that actin activated the catalytic loop of IR autophosphorylation in a concentration dependent manner (Fig. 5D).

3.6. Actin enhanced the autophosphorylation of the IR in the kinase domain

To determine whether actin regulates the autophosphorylation of the IR, we examined the effect of actin on the autophosphorylation of the kinase domain. Expression of the cytoplasmic domain of the IR in the absence of α -subunits has been shown to be constitutively active and the protein autophosphorylates [31]. We used the constitutively active cytoplasmic domain of the IR [32] and measured the IR kinase activity in the presence and absence of actin. The results indicate that actin enhanced the kinase activity of the IR (Fig. 6A), suggesting that actin may directly act on the kinase domain. Previously, actin has been shown to be phosphorylated by IR [33;34]. To make sure that IR activation by actin is true activation of IR and is not due to the phosphorylation of actin (CPM we obtained in the assay includes peptide phosphorylation and actin phosphorylation by the IR), we carried out the IR kinase activity assay without peptide but in the presence and absence of actin. The results indicate the absence of phosphorylation of actin and the IR kinase activity is identical in the presence and absence of actin (Fig. 6A). We failed to observe the phosphorylation of actin (Fig. 6A). These results suggest that actin enhanced the IR kinase activity.

Actin exists in two structural forms and each isoform can take either a globular or filamentous form [35]. To determine which structural form regulates the IR activation, purified G-actin and F-actin were incubated with constitutively active cytoplasmic domain of the IR [32] and the IR kinase activity was measured. Incubation with G-actin resulted in a significant increase in the IR kinase activity (Fig. 6B). F-actin failed to activate the IR kinase activity and incubation of IR kinase with 100 mM KCl (KCl facilitates the polymerization of G-actin to F-actin) did not affect the IR kinase activity (Fig. 6B). These results indicate that actin and its polymerization state play an important role in the regulation of the IR kinase activity.

To further confirm that G-actin activates IR autophosphorylation, two actin mutants were generated by SDM. S14C-actin (favors F-actin formation and increases the F-actin/G-actin

ratio) and R62D-actin mutant (unable to polymerize and decreases F-actin/G-actin ratio) proteins were expressed by *in vitro* coupled transcription and translation. The reaction products were subjected to Western blot analysis with anti-His antibody, and the results verified the expression of wild-type and mutant actin proteins (Fig. 7A). The IR immunoprecipitates were subjected to *in vitro* phosphorylation in the presence of wild-type, S14C and R62D mutant actins, followed by Western blot analysis with the anti-PY99 antibody (Fig. 7B). The blot was stripped and reprobed with anti-IR β antibody to ensure equal amounts of IR in each lane (Fig. 7C). The results indicate that the S14C-actin mutant decreased the autophosphorylation of the IR, suggesting that G-actin favors the autophosphorylation of retinal IR.

3.7. Reduced binding of actin with the IR under hyperglycemia

A decrease in the retinal IR kinase activity after 4 weeks of hyperglycemia has recently been reported [14]. We examined whether the decreased IR kinase activity is correlated with actin binding to the IR. The mean blood glucose levels of citrate-buffered controls and STZ-injected mice are given in Fig. 8A. The IR was immunoprecipitated from control and hyperglycemic mouse retinas and the immune complexes were subjected to Western blot analysis using anti-actin antibody. To ensure equal amounts of IR in each immunoprecipitation we reprobed the blot with anti-IR β antibody. Densities were calculated from the respective immunoblots and the results are expressed as actin/total IR. The results indicate a decreased actin association with retinal IR in hyperglycemic mice compared to control mice (Fig. 8B). These results suggest that the reduced IR kinase activity reported [14] previously might be due to the decreased association of actin with the IR.

3.8. Rhodopsin measurement in control and STZ mice

A growing body of evidence suggests that the neural retina undergoes significant deterioration early in the course of diabetes [36;37]. In humans, this evidence includes altered electroretinograms, diminished color vision, and contrast sensitivity before the clinical diagnosis of diabetic retinopathy [36;37]. The apoptotic death of photoreceptors in diabetic animal models has been demonstrated [38], and photoreceptor changes have been reported in patients with diabetic retinopathy [39]. We have recently reported that photobleaching of rhodopsin regulates the phosphorylation of the retinal IR [9]. The loss of IR activation in STZ mice prompted us to think that there could be a defect in the photobleaching of rhodopsin. To rule out this possibility, we measured the rhodopsin content in the citrate buffered control and the STZ-treated mice. The results indicate no difference in the rhodopsin content between control and STZ treated mice (Fig. 9A). To further confirm this result we prepared ROS from control and STZ mice, and the ROS proteins were subjected to SDS-PAGE followed by GelCode blue staining. The results indicate no difference in the expression of photoreceptor specific proteins such as phosphodiesterase (PDE), arrestin, transducin alpha subunit, and opsin between control and STZ treated mice (Fig. 9B). Collectively, these results suggest that the loss of IR activation in STZ mice is not due to a defect in the photobleaching of rhodopsin.

4. Discussion

The central result of the present investigation is that cytoplasmic actin associates with and activates retinal IR autophosphorylation *in vivo*. Further, the binding of actin to the IR is reduced under hyperglycemic conditions. Our studies suggest that G-actin favors the activation of IR and, also, the state of actin polymerization modulate the kinase activity of retinal IR. The retinal IR autophosphorylation [28] and retinal IR kinase activity [14] is shown to be decreased in diabetes. Results from our study indicate that the binding of actin to the IR is reduced in diabetes and this loss of interaction may result in reduced IR activation in diabetic retinopathy [14]. We have reported previously that photobleaching of rhodopsin is required for the activation of the retinal IR [9]. In 4 weeks of diabetes we did not observe any change in the

photobleaching of rhodopsin; however, we did observe a decreased binding of actin to the IR. The loss of IR activation [28] and reduced binding of actin to IR in diabetes could be due to the modification of cytoplasmic actin by glycation and/or oxidation. In the brain and lung, actin is modified by glycation in experimental diabetes as demonstrated by labeling with the anti-glucitolysine antibody [40]. Actin is also known to be modified by 4-hydroxy-2-nonenal (HNE) and carbonylation [41;42]. Further, methionine oxidation is a major cause of the functional impairment of oxidized actin [43;44]. Further studies, however, are required to examine whether actin modification has a role in the autophosphorylation of retinal IR.

Cytoskeletal boundary protein and the plasma membrane control cell shape, delimit specialized membrane domains and stabilize attachments to other cells and to the substrate [45;46]. The IRs in the retina, especially in the rod outer segments, are localized to plasma membrane [9]. This suggests that IR is in close association with the actin cytoskeleton. It is not clear how the high extracellular glucose effects the IR/actin association inside retinal cells during hyperglycemia. However, it has previously been shown that high glucose alters the physical properties of the extracellular matrix through the non-enzymatic glycation of proteins, leading to changes in the organization of the intracellular actin cytoskeleton [47]. Additionally, dysfunction of the actin cytoskeleton is a key event in the pathogenesis of diabetic nephropathy [48], diabetic neuropathy [49;50] and diabetic cardiomyopathy [51;52]. The IR/actin association we observed in this study could be an important element in the control of diabetic retinopathy.

The molecular mechanism behind the actin-induced activation of the IR is not known. In this study we observed the recognition of both insulin and actin-induced autophosphorylation of IR by phosphospecific IR antibody which specifically recognizes the tyrosine phosphorylation in the catalytic loop of IR at tyrosine 1158, 1162 and 1163. Further, actin also enhanced the kinase activity of the cytoplasmic domain of the IR. These results clearly suggest that actin activates the tyrosine phosphorylation in the catalytic loop of IR. Further studies are required to understand the interaction and occupancy of actin on retinal IR.

Actin exists in two interchangeable isoforms [53] that can take either a globular or filamentous form [35]. Globular actin (G-actin) readily polymerizes under physiological conditions to form filamentous actin (F-actin) with the concomitant hydrolysis of ATP [35]. In this study we observed that G-actin favors the activation of the IR *in vitro*. It has been shown previously that the association of endothelial nitric oxide (eNOS) synthase with G-actin causes a more significant increase in eNOS activity than caused by association with F-actin [54]. In cells, the assembly and disassembly of actin filaments and their organization into functional higher order networks is regulated by a plethora of actin-binding proteins [53]. The activities of these proteins are, in turn, under the control of specific signaling pathways [55].

In this study we have demonstrated that actin interacts with the retinal IR. These results also suggest that the retinal IR may have an actin binding site(s). It has previously been shown that the EGF receptor is an actin-binding protein [56]; furthermore, the actin-binding domain of the EGF receptor is required for EGF-stimulated tissue invasion [57]. Molecular cloning of the retinal IR is important to further characterize the actin-induced autophosphorylation of the retinal IR. Further studies, however, are necessary to determine whether the state of actin polymerization represents a valid target for therapy in diabetic retinopathy. These studies are important for understanding the mechanism(s) of diabetic retinopathy and to identify targets for future therapeutic intervention.

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ABBREVIATIONS

IR	insulin receptor
PI3K	phosphoinositide 3-kinase
IRβ	IR beta subunit
PAGE	polyacrylamide gel electrophoresis
STZ	streptozotocin
ROS	rod outer segments
IPs	immunoprecipitates
G-actin	globular actin
F-actin	filamentous actin
SDM	site-directed mutagenesis

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Figure 1.

Autophosphorylation of IR in the presence of non-receptor tyrosine kinase inhibitors. The IR was immunoprecipitated with anti-IR β antibody from retinal lysates (250 μ g) and subjected to phosphorylation in the presence of 100 μ M PP1, PP2 and PP3 followed by Western blot analysis with anti-PY99 (A) and anti-IR β (B) antibodies. Bovine ROS was subjected to *in vitro* phosphorylation in the presence of 100 μ M PP1, PP2 and PP3. The reaction products were subjected to SDS-PAGE followed by Western blot analysis with anti-PY99 (C) and anti-arrestin (D) antibodies.

Figure 2. Identification of a 42 kDa protein associated with retinal IR. The IR was immunoprecipitated from the retinal lysates (250 µg) with anti-IRβ, and the bound proteins were resolved by SDS-PAGE. The arrow indicates the location of the 42 kDa protein. Lane 1, 10 µg retinal lysate; lane 2, IRβ immunoprecipitate.

Figure 3. Co-immunoprecipitation of IR and actin. Retinal lysates were subjected to immunoprecipitation with anti-IR β antibody or non-immune IgG followed by Western blot analysis with anti-actin (A) and anti-IR β antibody (B).

Figure 4.

Interaction of actin and IR. Varying amounts of retinal and liver proteins were subjected to Western blot analysis with anti-IR (A) and anti-actin (B) antibodies. Densities of IR and actin were measured and IR was normalized to actin (C). The IR was immunoprecipitated with anti-IR β antibody (B) from retinal and liver lysates. The immune complexes were subjected to Western blot analysis with anti-actin antibody (D). The blot was reprobbed with anti-IR antibody to ensure equal amounts of IR in each immunoprecipitate (E). Densities of IR and actin were measured and IR was normalized to actin (F). Data are mean $\pm$ SD, n=4. The IR immune complexes from retina and liver were subjected to *in vitro* phosphorylation in the presence 4.6 or 9.3 μ M actin. The phosphorylation reaction was subjected to Western blot analysis with PY-99 antibody (G). The blot was reprobbed with IR to ensure equal amounts of protein in each immunoprecipitate (H).

Figure 5.

Actin-induced activation of the retinal IR. The IR was immunoprecipitated with anti-IR β antibody (C) from retinal (500 μ g) lysates. The immune complexes were subjected to *in vitro* phosphorylation in the presence of 1 μ M insulin or 9.3 μ M actin. The phosphorylation reaction was subjected to Western blot analysis with phosphospecific anti-IR (pYpYpY^{1158/1162/1163}) antibody (A). The added actin was examined with anti-actin antibody (B). The IR immunoprecipitates (E) were subjected to *in vitro* phosphorylation in the presence of varying concentrations of actin for 60 min at room temperature. The phosphorylation reaction was subjected to Western blot analysis with anti-pIR (pYpYpY^{1158/1162/1163}) antibody (D).

Figure 6.

Effect of actin on the IR kinase activity. Purified IR kinase domain was used to phosphorylate either actin or poly (Glu:Tyr) substrate or phosphorylation of poly (Glu-Tyr) substrate in the presence of actin (A). Purified IR kinase domain was incubated with poly (Glu:Tyr) substrate and the IR kinase activity was measured in the presence of either 100 mM KCl or 9.3 μM G-actin or 9.3 μM F-actin (B). The control experiment was carried out in the absence of additives. The reaction was initiated by the addition of [γ ^{32}P]ATP. The IR kinase activity was expressed as $\mu\text{mol}/\text{min}/\text{mg}$ protein. Data are mean $\pm$ SD, $n=5$. One-way ANOVA with unpaired t-tests were used for assessing significant differences across groups. Significance for Fig. 6A: Groups 1 vs 2, $p<0.42$; Groups 1 vs 3, $p<0.0079$; Groups 1 vs 4, $p<0.001$; Groups 2 vs 3, $p<0.0079$; Groups 2 vs 4, $p<0.015$; Groups 3 vs 4, $p<0.032$. Significance for Fig. 6B: Groups 1 vs 2, $p<0.68$; Groups 1 vs 3, $p<0.009$; Groups 1 vs 4, $p<0.62$; Groups 2 vs 3, $p<0.0014$; Groups 2 vs 4, $p<0.85$; Groups 3 vs 4, $p<0.0066$.

Figure 7.

Effect of wild-type and actin mutants on the activation of retinal IR. Empty vector (EV), pCDNA3 vector carrying Wild-type (WT) or S14C or R62D mutant actins were used for the synthesis of proteins using *in vitro* coupled transcription and translation. The *in vitro* synthesized proteins were subjected to Western blot analysis with anti-His antibody (A). The IR immunoprecipitates (250 μ g) were subjected to *in vitro* phosphorylation in the presence of wild-type, S14C and R62D mutant actins, followed by Western blot analysis with the anti-PY99 antibody (B). The blot was stripped and reprobed with anti-IR β antibody to ensure equal amounts of IR from each immunoprecipitate (C).

Figure 8.

Interaction between actin and IR under hyperglycemia. Blood was drawn from control and STZ-treated mice from the tail vein and the blood glucose levels were monitored with a glucometer (A). Data are mean $\pm$ SD, $n=4$, $*p<0.001$. The IR was immunoprecipitated with anti-IR β antibody from retinal lysates (250 μ g) of control and STZ mice followed by Western blot analysis with anti-actin antibody. Densities were calculated from the respective immunoblots and the results are expressed as actin/total IR (B). Data mean $\pm$ SD, $n=4$. Unpaired t test was used to compare the significance between control and STZ, $p<0.06$.

Figure 9.

Determination of rhodopsin content from citrate buffered control and STZ mouse retinas. Rhodopsin content was measured by spectrometry from control and STZ mouse retinas. Data are mean $\pm$ SD, n= 5. ROS was prepared from 5 control and 5 STZ mice. Five micrograms of ROS membranes were subjected to SDS-PAGE followed by GelCode blue staining. Position of phosphodiesterase (PDE), arrestin, transducin alpha subunit and opsin are shown.

Table 1

Mass Spectrometric Analysis of 42 kDa Protein

Start-End	Observed	Mr (expt) Da	Mr (calc) Da	Sequence
96–113	1953.93	1952.92	1953.06	VAPEEHPVLLTEAPLNPK
197–206	1132.42	1131.41	1131.52	GYSFTTTAER
239–254	1790.73	1789.72	1789.88	SYELPDGQVITIGNER
360–372	1516.67	1515.66	1515.70	QEYDESGPSIVHR

Four peptide fragments were identified by mass spectrometric analysis. Location of these fragments on the actin sequence as well as the observed, experimental and calculated masses were denoted in the table.