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Expression and purification of functional insulin and insulin-like growth factor 1 holoreceptors from mammalian cells

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Abstract

The insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF1R) are receptor tyrosine kinases (RTKs) involved in the regulation of many important cellular processes. The current proposed models of activation are derived from structural studies using soluble extracellular domains and cytoplasmic tyrosine kinase domains. Preparations of full length IR and IGF1R have been hampered by the need for unconventional affinity chromatography resins and/or harsh eluting conditions. Here, we present a purification protocol to obtain full-length, detergent solubilized IR and IGF1R at quantities suitable for biochemical and structural characterization. We screened a panel of 24 structurally diverse detergents for optimal ligand activation. The receptors purified in n-dodecyl- β -D-maltoside showed ligand-stimulated autophosphorylation and kinase activity, suggesting an intact transmembrane signaling mechanism. This convenient purification protocol can be used to produce high quantities of IR, IGF1R, or other RTKs, and can be adapted for other challenging membrane proteins.

Keywords

Insulin receptor; Insulin-like growth factor 1 receptor; Receptor tyrosine kinases; Streptavidin-binding peptide; Membrane proteins; Detergents

Introduction

Receptor tyrosine kinases (RTKs) mediate numerous cellular processes including cell differentiation, proliferation, and migration. RTKs share a similar overall structure that consists of an extracellular ligand binding domain, a transmembrane region, and an intracellular tyrosine kinase domain. There are 58 known human RTKs that can be divided into 20 families [1]. The insulin receptor family contains insulin receptor (IR), insulin-like growth factor-1 receptor (IGF1R) and insulin receptor-related receptor (IRR) [1]. The IR family receptors are structurally unique when compared to other RTKs; fully processed IR, IGF1R, and IRR are disulfide-linked preformed dimers, as opposed to monomers that dimerize upon activation [2–5]. IRR is the least understood within the IR family and has no known ligand, although IRR may act as an extracellular alkali sensor [6]. On the other hand,

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IR and IGF1R have been extensively studied because of their importance in the regulation of many normal cellular functions, and because of their roles in diseases such as diabetes and cancer [7,8]. Understanding the basic mechanism of activation of these receptors, and obtaining structural information, may aid in the design and development of treatments for diseases that are primarily caused by dysregulation of IR and IGF1R [9].

A direct approach to study the signal transduction mechanism of IR and IGF1R would be to isolate full-length, functional receptors. The currently proposed models of activation for IR and IGF1R are primarily derived from biochemical and structural data collected from studies of soluble ectodomains and kinase domains [10–16]. IR, IGF1R, and many other eukaryotic membrane proteins are challenging to express, extract, and purify at quantities required for structural analysis [17]. Despite recent advances in membrane receptor purification strategies [18], simple purification protocols for full length IR and IGF1R have been lacking. Full length IR was first purified from liver cell membranes using insulin linked Sepharose resin [19]. The purification of IR was improved by the development of methods such as wheat germ agglutinin (WGA)-agarose and anti-IR antibody conjugated agarose [20–30]. IGF1R was first partially purified from human placenta [31]. The purification protocols developed for IR were adapted for IGF1R, and IGF1R was isolated through the use of WGA-Sepharose, anti-IGF1R antibody conjugated Sepharose, and IGF1 conjugated Sepharose [32–37]. These purification methods required large amounts of materials and reagents, and typically required multiple purification steps and/or harsh elution methods.

There are unique challenges to the expression and purification of IR and IGF1R which arise from the quaternary structure of these receptors. IR and IGF1R are expressed as $\alpha\beta$ precursors that require processing and cleavage by furin [2,4]. They are also heavily glycosylated in their extracellular domains [38,39]. These processing steps dictate that eukaryotic expression systems must be used for the receptors. Overexpression of IR in *Spodoptera frugiperda* (Sf9) insect cells using a baculovirus vector resulted in the accumulation of precursor [30], suggesting that the levels of expression cannot exceed the capacities of the endogenous cellular processing machinery. Additionally, since disulfide bonds are integral to the final form of the receptors, reducing agents (usually used to prevent protein oxidation) must be omitted from the purification protocol [40]. In order to preserve native transmembrane signaling, it is essential to purify fully processed and properly disulfide bonded forms of the receptors.

We present a single step affinity chromatography protocol to purify full length, detergent soluble, active forms of IR and IGF1R at significant quantities for biochemical and structural characterization. We cloned receptor cDNAs into mammalian expression vectors that can be easily manipulated by site-directed mutagenesis for future studies. We generated stable human embryonic kidney (HEK) 293 T cell lines expressing IR and IGF1R to ensure native like processing and to reduce the accumulation of the $\alpha\beta$ precursor. We demonstrated ligand-stimulated autophosphorylation and activation of the receptors. We also screened 24 structurally diverse detergents to determine which conditions best preserved IR activity and ligand responsiveness.

Materials and methods

Vectors

Full details of the construction of the IR and IGF1R expression plasmids are provided in the accompanying Supplementary Information. Briefly, human IR and IGF1R cDNA (including signal peptide sequences) were amplified from plasmids pBPV-IR and pBPV-IGF1R [41,42] using PCR. Restriction sites were introduced and were used to ligate the cDNAs into a modified pCMV-HA vector (Clontech) containing an *AscI* restriction site immediately 5' to the hemagglutinin (HA) tag. IR was cloned into pCMV-HA as an *AscI/SaII* fragment, and IGF1R as an *AscI/EcoRI* fragment. A streptavidin-binding peptide (SBP)-tag [43] (kind gift from Dr. Takashi Murayama [44]), three amino acid linker, six histidine residue (His)-tag, and stop codon were added after residue Glu1259 for IR and Cys1337 for IGF1R using the protocol from the QuikChange II Site-Directed Mutagenesis Kit (Agilent), modified for large inserts [45].

Western blotting

All samples were analyzed by SDS-PAGE on 8% acrylamide gels and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore). Primary antibodies used were anti-SBP-tag (Millipore, MAB10764, Clone 20, 1:1000 dilution), Anti-IR α (Santa Cruz, sc-7953, H-78, 1:500 dilution), Anti-IR β (Cell Signaling, #3025, 4B8, 1:1000 dilution), Anti-IGF1R α (Santa Cruz, sc-271606, G-5, 1:1000 dilution), Anti-IGF1R β (Cell Signaling, #14534, D4O6W, 1:1000 dilution), Anti-pYpY (IR phosphorylated Tyr^{1162/1163} and IGF1R phosphorylated Tyr^{1135/1136}, Calbiochem, 407707-10 T, 1:1000 dilution). Secondary antibodies used were horseradish peroxidase conjugated antibodies of either rabbit IgG (GE Healthcare Life Sciences, 1:10,000 dilution) or mouse IgG (GE Healthcare Life Sciences, 1:10,000 dilution). Lastly, membranes were incubated with Western blotting substrate (Thermo Scientific Pierce ECL) and exposed to autoradiographic film (BioExcell).

Generation of stable HEK 293 T cells

Human embryonic kidney (HEK) 293 T cells (ATCC, CRL-3216) were seeded at a density of 1×10^6 cells in a 10 cm tissue culture dish with 10 mL standard media consisting of 1X Dulbecco's Modification of Eagle's Medium (DMEM, Corning, 10-013-CV) with 5% (v/v) fetal bovine serum (VWR Life Science Seradigm) and 1X antibiotic-antimycotic solution (Corning). One day after seeding, cells were co-transfected with 1 μ g pBABE-PURO [46] and 10 μ g of pCMV-IR or pCMV-IGF1R. After 48 h, transfected cells were treated with 2 mL 1X trypsin EDTA (Corning), quenched with 8 mL media, and split by diluting 1:10, 1:5, and 1:2 into three individual 10 cm dishes with standard media containing 1 μ g/mL puromycin (Gibco). Media with puromycin was changed 24 h after initial selection and every 3–4 days until visible colonies formed. Colonies were isolated with cloning cylinders (Corning) and transferred to 24-well tissue culture plates. Colonies were maintained in 24-well plates, and expression of receptors was checked by anti-SBP-tag Western blotting. The colonies with the highest expression of either IR or IGF1R were expanded from 24-well plates to 6-well plates by splitting cell suspensions at a 1:10 dilution and allowing the cells to reach ~90% confluency. This process was repeated to expand colonies from 6-well plates to 10 cm dishes. For receptor purifications, eight 15 cm dishes were individually seeded with

3×10^6 cells and grown to confluency (approximately 3–4 days). Cells from the 15 cm dishes were harvested by pipetting 1X phosphate-buffered saline (Corning) to dislodge the cells. Cell suspensions were combined and centrifuged at $1000 \times g$ for 5 min at 4 °C in a swinging-bucket centrifuge. Supernatant liquid was removed and cell pellets were stored at –80 °C.

Receptor purification by affinity chromatography

Purification of receptors was performed at 4 °C unless otherwise noted. A single cell pellet was thawed for 30 min and lysed for 1 h with 40 mL of Lysis Buffer (20 mM Tris, pH 8, 400 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 5 µg/mL leupeptin, and 5 µg/mL aprotinin) on end-over-end rocker. Lysate was centrifuged for 30 min at $12,520 \times g$ and the supernatant liquid was filtered through 0.8 µm syringe filters (Millex-AA, Millipore). A total of 5 mL packed Strep-Tactin Superflow Plus resin (Qiagen) was equilibrated with 10 mL Lysis Buffer, combined with cleared lysate, and incubated for 1 h on end-over-end rocker.

Resin was poured into a 1.5 cm diameter glass chromatography column and rinsed with 15 mL Buffer A (20 mM Tris, pH 8, 200 mM NaCl, 10% glycerol, 0.1% Triton X-100). Detergent was exchanged to n-Dodecyl-β-D-maltoside (DDM) by the addition of 30 mL Buffer B (20 mM Tris, pH 8, 200 mM NaCl, 10% glycerol, 0.03% DDM). Receptor was eluted in a single 10 mL fraction with Elution Buffer (Buffer B containing 2.5 mM d-desthiobiotin). Eluate was concentrated to ~1 mL using a stirred cell nitrogen concentrator (Millipore, Model 8010) with a 10 kDa NMWL membrane (Millipore, Ultracel).

Residual tyrosine phosphorylation was removed by incubation with *Yersinia* tyrosine phosphatase (YOP). Glutathione S-transferase (GST) tagged YOP was immobilized on glutathione agarose (Invitrogen) and washed with 50 mM Tris (pH 8). The glutathione agarose resin contained ~0.8 mg GST-YOP per 1 mL packed resin. For the removal of residual phosphotyrosine, immobilized GST-YOP (100 µL packed resin) was pre-equilibrated with Elution Buffer, and incubated for 20 min with concentrated receptor (~1 mL, 100–500 µg/mL receptor) in Elution Buffer on an end-over-end rocker. The immobilized GST-YOP was removed by centrifugation at $1000 \times g$. Protein concentrations were determined using the Bio-Rad dye-binding assay. Concentrated receptor was aliquoted and stored at –80 °C.

Gel filtration of purified receptors

A Superose 6 Increase 10/300 GL column (GE Healthcare) was equilibrated at 4 °C with Buffer C (20 mM Tris, pH 8, 200 mM NaCl, and 0.03% DDM) at a flow rate of 0.5 mL/min. A calibration trace was generated by loading a 500 µL sample containing 0.6 mg thyroglobulin, 0.15 mg apoferritin, 0.6 mg thyroglobulin, and 0.6 mg carbonic anhydrase (Sigma) in Buffer C. IR and IGF1R receptors (500 µL) were loaded onto the column and 1 mL fractions were collected for analysis by Western blotting.

Purification of secreted, soluble form of human furin (ss-furin)

Full details of the construction of the ss-furin expression plasmid is provided in the accompanying Supplementary Information. The overall strategy was similar to Bravo et al.

[47] with minor changes. Human furin cDNA was amplified by PCR from plasmid pVL1392 (kind gift from Dr. Claire Dubois [48]). The furin cDNA was cloned into pFastBacHTc (Invitrogen) using 5' *EcoRI* and 3' *NoI* sites, and a His-tag was added after codon 595. Generation of baculovirus and expression of ss-furin was carried out using the Bac-to-Bac system (Invitrogen) according to the manufacturer's instructions. Cells were removed from media by centrifugation for 5 min at $12,520 \times g$. Media containing ss-furin was harvested and either purified immediately or stored at -80°C for subsequent purifications.

Purification of ss-furin was carried out using 3 mL Ni-NTA agarose resin (Qiagen) equilibrated with 10 mL Buffer D (20 mM Tris, pH 8, 400 mM NaCl, 10% glycerol, 1 mM CaCl_2). Media containing ss-furin (45 mL) was passed over the column. The column was washed extensively with 60 mL Buffer D containing 20 mM imidazole, and ss-furin was eluted in 1 mL fractions with Buffer D containing 200 mM imidazole. Fractions with ss-furin were concentrated using a stirred cell nitrogen concentrator with a 10 kDa NMWL membrane. Concentrated ss-furin was further purified on a Superose 12 10/300 GL column (GE Healthcare) equilibrated with Buffer D. Peak fractions were collected, concentrated, and stored at -80°C .

Precursor treatment with ss-furin

Reactions containing 150 $\mu\text{g/mL}$ IR or 80 $\mu\text{g/mL}$ IGF1R with 0.8 $\mu\text{g/mL}$ ss-furin in Buffer B containing 2 mM CaCl_2 . After incubation at 30°C for 2 h, samples were quenched with 5X Laemmli buffer. Samples were analyzed by SDS-PAGE on 10% acrylamide gels and visualized by Coomassie Blue staining.

Autophosphorylation assay

Autophosphorylation reactions had 15 $\mu\text{g/mL}$ purified receptor with or without 200 nM of appropriate ligand (insulin or IGF1) in a Buffer C. Reactions were incubated for 10 min at room temperature before the addition of 10X Kinase Buffer A (10 mM ATP, 10 mM MgCl_2 in Buffer C). Reactions were quenched at various time points by the addition of 5X Laemmli buffer and analyzed by Western blotting.

Detergent screen assay

HEK 293 T cells expressing IR were lysed, and receptors were bound to Strep-Tactin Superflow Plus resin and washed with Buffer A as described in section 2.4. Receptor bound resin was resuspended in a 1:1 (v/v) ratio to create a slurry. Aliquots (150 μL) of slurry were centrifuged for 1 min at $1000 \times g$, supernatant was removed, and resins were washed with 100 μL aliquots of Detergent Exchange Buffer (20 mM Tris, pH 8; 200 mM NaCl) containing various detergents (Jena Bioscience, JBScreen Detergents 1, CS-521) at 1.25X critical micelle concentration (CMC) a total of three times. Receptors were eluted by incubation with 100 μL Detergent Exchange Buffer containing 5 mM d-desthiobiotin for 5 min at room temperature, and resin was removed by centrifugation. For each detergent condition, eluted receptor was split into two 40 μL aliquots. Duplicate autophosphorylation reactions with or without insulin (200 nM) were initiated by the addition of 10X Kinase Buffer B (10 mM ATP, 10 mM MgCl_2). Final reaction volumes were 50 μL , and detergents

were at 1X CMC. Reactions were quenched by the addition of 5X Laemmli buffer after 5 min and analyzed by Western blotting. Developed films were scanned and band intensities were quantified using ImageJ [49].

Radiometric kinase assay

Batch reactions (150 μ L final volume) contained 15 μ g/mL receptor, 10 mM MgCl_2 , 0.5 mM ATP, 1–6 μCi [γ - ^{32}P]-ATP (PerkinElmer, 10 mCi/mL, 25–50 cpm/pmol) and synthetic peptide KEEEEYMMMMG at concentrations of 0.05 mM–2 mM in a Buffer B. Some reactions contained insulin or IGF1 at concentrations of 200 nM. Reactions were stopped at various times by removing 25 μ L from the batch reaction and quenched by adding to 45 μ L cold 10% (w/v) trichloroacetic acid. Samples were centrifuged at $10,000 \times g$ for 2 min and 35 μ L of supernatant was spotted on P81 phospho-cellulose paper (Whatman). Excess radiolabeled ATP was removed by three 10 min washes in cold 0.5% phosphoric acid followed by a final wash in acetone. The P81 paper circles were dried and activity in counts per min (cpm) was measured in a scintillation counter.

Continuous enzyme-coupled assay

The assay protocol was modified from Barker et al. [50]. Briefly, reactions contained 150 μ g/mL IR, 20 mM Tris pH 8.0, 200 mM NaCl, 0.03% DDM, 10 mM MgCl_2 , 1 mM phosphoenolpyruvate, 1.4 mM NADH, 120 units pyruvate kinase, 1680 units lactate dehydrogenase, 0.25 mM synthetic peptide KEEEEYMMMMG, and 0.5 mM ATP. Stimulated IR reactions contained 1 μ M insulin. Absorbance was monitored at 340 nm in 96 well microplates (Corning).

Results and discussion

We generated stable human embryonic kidney (HEK) 293 T cell lines expressing human IR and IGF1R (Fig. 1a–b). We selected a mammalian cell line because we wished to preserve native like processing of the receptors (glycosylation, cleavage of signal peptide, cleavage of the precursor, and proper assembly of the full length $\alpha_2\beta_2$ heterotetramer). We expected that the processing steps and the membrane environment in HEK 293 T cells would be more physiologically relevant to the study of human receptors than (for example) insect cells. The generation of stable cell lines also had technical advantages over transient transfections for the expression and purification of IR and IGF1R. It is less labor intensive to expand and harvest large batches of stable cells for purification than to conduct repeated transient transfections, and it eliminates the need for expensive transfection reagents. Furthermore, expression of the receptors was more reliable, with less batch-to-batch variability, than for transient transfections, where small differences in the protocol and in the reagents can result in altered efficiency and quality of the transfections. (Supplemental Fig. S1 shows representative expression of IR and IGF1R from multiple batches of stable cells). Importantly for our studies, expression of IR and IGF1R in stable cell lines produced primarily the processed α and β subunits (Fig. 1a–b), whereas transient expression led to accumulation of $\alpha\beta$ precursor (Fig. 1c). This suggests that transient expression of IR and IGF1R taxes the endogenous cellular processing machinery. The accumulation of

unprocessed precursor was also observed when IR was overexpressed in Sf9 insect cells using a baculovirus vector [30].

To aid in the purification, we added SBP-tags [43] to the C-terminus of the β -subunits of IR and IGF1R. We purified receptors to near homogeneity in a single step protocol using Strep-Tactin resin and buffers containing DDM for stability. The Strep-Tactin eluate contained some unprocessed $\alpha\beta$ precursor which could be removed by treatment with ss-furin (Fig. 2). The processed α and β subunits are visualized by SDS-PAGE under reducing conditions (Fig. 2), and we confirmed their identities by Western blotting (Fig. 1). We were able to consistently obtain 100–500 μ g of full length, detergent solubilized IR or IGF1R. These levels were suitable for biochemical analysis.

To confirm that the α and β chains assembled into an $\alpha_2\beta_2$ heterotetramer, purified IR and IGF1R were analyzed on a Superose 6 gel filtration column (Fig. 3). The major peaks for IR and IGF1R eluted in the 550 kDa–650 kDa range. Predicted molecular weights from amino acid sequence alone are 301 kDa for IR and 298 kDa for IGF1R. Glycosylation adds significantly to the size of the receptors [38,39]. From the migration of individual subunits under reducing conditions (Fig. 2), receptors in the $\alpha_2\beta_2$ heterotetrameric form would have a molecular weight of approximately 450 kDa. The receptors were purified from HEK 293 T cells using the detergent DDM; a single micelle of DDM has a molecular weight of approximately 72 kDa [51]. With each fully assembled receptor, there are two transmembrane domains stabilized by DDM micelle(s) that would add to the molecular weight. The receptors are also not symmetrical spheres, adding to the apparent molecular weights when analyzed on a gel filtration column [52]. Given these considerations, the behavior of IR and IGF1R on gel filtration are consistent with the existence of detergent-solubilized, $\alpha_2\beta_2$ heterotetramers. This was confirmed by activity measurements (see below), as the isolated α and β subunits would not be responsive to insulin or IGF1. For some experiments that have required higher purity, we conducted preparative separation of the receptors using gel filtration.

Next, we screened a series of detergents to identify conditions that would preserve transmembrane signaling by the receptors. Most common detergents are classified as ionic, non-ionic, or zwitterionic. Ionic detergents tend to solubilize membrane proteins well, but can have a denaturing effect. Non-ionic detergents are considered milder for isolation of membrane proteins, although some short chained non-ionic detergents have resulted in loss of activity. Zwitterionic detergents have been used in structural studies of membrane proteins, but they also tend to result in deactivation [53]. Therefore, we screened IR autophosphorylation activity with and without insulin with 24 structurally diverse detergents (Fig. 4, Table 1). In these experiments, we measured IR autophosphorylation by Western blotting with antibodies specific for pY1162/pY1163. These tyrosine residues are within the activation loop; phosphorylation occurs in parallel with a large increase in kinase activity [54,55]. Our goal was to identify detergents that met two conditions: (1) low IR autophosphorylation activity in the basal (unstimulated) state, and (2) high autophosphorylation activity when stimulated with insulin. Several detergents (Anapoe-NID-P40, 1,2-diheptanoyl-sn-glycero-3-phosphocholine, Anapoe-C10E9, MEGA-10, and n-undecyl-N,N-dimethylamine-oxide) gave high insulin-stimulated IR activity, but also led to

IR autophosphorylation in the basal state. These results suggest that the IR autoinhibitory contacts are not preserved in these detergents, leading to unregulated kinase activity (Fig. 4). The three detergents that gave the highest degrees of insulin stimulation were DDM, C12E8, and n-decyl- α -D-maltoside (Table 1). This followed a trend seen in other membrane proteins, in which activity is preserved with non-ionic detergents with an intermediate chain and lost with short chain derivatives [53]. DDM has also been shown to be a good detergent for preserving activity in the epidermal growth factor receptor [56]. For all subsequent biochemical assays of IR and IGF1R, we chose to use DDM as the detergent for solubilization and stabilization.

We followed the time course of ligand-stimulated IR and IGF1R activation. Both receptors showed a marked increase in activation loop phosphorylation over basal levels when incubated with their cognate ligands (Fig. 5). The rate of basal autophosphorylation in these experiments was higher than in Fig. 4 because of the higher protein concentrations and longer time course. The degree of activation in our experiments with purified receptors (~10-fold) is comparable to the activation we observe in intact IR-expressing HEK293T cells (Supplementary Fig. S2), and to that observed in intact rat liver [57], brain slices [58], or in other mammalian cells (e.g., 11-fold in Moller et al. [59]). This demonstrates that the ligand-induced signal transduction mechanism is still operable in our detergent-solubilized IR and IGF1R preparations.

We confirmed the kinase activity of IR and IGF1R using a radiometric assay that measures phosphorylation of a synthetic peptide substrate (Fig. 6) [60]. The peptide was based on results from a peptide library screen of IR specificity [61]. Both receptors were active toward this substrate, and activity increased in the presence of ligand (insulin or IGF1). We obtained similar results using a continuous spectrophotometric kinase assay (Fig. 6b). The receptors were also inhibited by linsitinib (OSI-906), a selective inhibitor of IR and IGF1R [62] (Fig. 6). There was a smaller increase in kinase activity in the peptide assays after stimulation (~2 fold) as compared to the autophosphorylation assays. This is most likely due to receptor autophosphorylation and activation during the course of the peptide assays, which are done in the presence of ATP. We also showed that purified IR is relatively stable in DDM detergent buffer, since no difference in phosphorylation of a peptide substrate was detected after incubation at 4 °C for 24 h (Fig. 6b).

We carried out steady state kinetic measurements toward peptide substrate using the radiometric phosphorylation assay (Fig. 7, Table 2). The values of K_m for this substrate were 235 μ M and 386 μ M for IR and IGF1R, respectively. The V_{max} of IGF1R was approximately double that of IR. The specific activity of this preparation of IR (171 nmol/min/mg) was comparable to previously reported values for IR purified by other methods. Petruzzelli et al. measured IR specific activity to be 80 nmol/min/mg towards angiotensin [25]. The IR preparation from Pike et al. used a different peptide substrate (RRLIEDAEYAARG) and had a specific activity of 117 nmol/min/mg in the absence of insulin and 300 nmol/min/mg in the presence of insulin [63]. A preparation of the isolated IGF1R kinase domain had a specific activity of 2.1 μ mol/min/mg [64]. IGF1R was recently purified by monoclonal antibody linked resin [37]. The specific activity was not reported, but the receptor had an apparent K_m of 66 μ M and k_{cat} of 62 min⁻¹ for a biotinylated synthetic peptide [37].

Conclusions

Bacterial systems are frequently used to generate high quantities of proteins for biochemical and structural studies. However, prokaryotic systems are not typically suitable for production of mammalian membrane proteins because they lack the machinery for proper processing and post-translational modification. Here, we report a protocol for expression and purification of full length, detergent solubilized IR and IGF1R from mammalian cells at quantities suitable for biochemical assays. A major basis for the success of the method was the introduction of the SBP-tag, which binds streptavidin with a K_d of 2.5 nM [43]. Both IR and IGF1R are stimulated by their cognate ligands (insulin or IGF1), resulting in increased autophosphorylation and substrate phosphorylation, suggesting that the transmembrane signaling mechanism is intact.

Membrane proteins are underrepresented in structural databases. A major hurdle for the characterization of membrane proteins is expression and purification. The $\alpha_2\beta_2$ structures of IR and IGF1R have made purification particularly challenging. Here, we report procedures for expression and purification of functional IR and IGF1R in quantities high enough for structural studies such as single particle electron microscopy and cryo-EM. The protocol can be scaled up to generate larger amounts of purified receptors for membrane crystallography. Detergents could be substituted for other membrane protein stabilization agents including lipid-detergent micelles, amphipols, and vesicles. An important feature of our heterologous expression method is that it can easily be adapted for production of mutant forms of IR and IGF1R. Furthermore, the protocol should be broadly applicable to other RTKs and mammalian membrane proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

RTKs	Receptor tyrosine kinases
IR	Insulin receptor
IGF1R	Insulin-like growth factor receptor
SBP	Streptavidin-binding peptide
DDM	n-Dodecyl- β -D-maltoside

CMC Critical micelle concentration

References

1. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell*. 2010; 141:1117–1134. [PubMed: 20602996]
2. Lawrence MC, McKern NM, Ward CW. Insulin receptor structure and its implications for the IGF-1 receptor. *Curr Opin Struct Biol*. 2007; 17:699–705. [PubMed: 17851071]
3. Ward CW, Lawrence MC. Similar but different: ligand-induced activation of the insulin and epidermal growth factor receptor families. *Curr Opin Struct Biol*. 2012; 22:360–366. [PubMed: 22521506]
4. Ward CW, Lawrence MC. Landmarks in insulin research. *Front Endocrinol (Lausanne)*. 2011; 2:76. [PubMed: 22654826]
5. Adams TE, Epa VC, Garrett TP, Ward CW. Structure and function of the type 1 insulin-like growth factor receptor. *Cell Mol Life Sci*. 2000; 57:1050–1093. [PubMed: 10961344]
6. Deyev IE, Mitrofanova AV, Zhevlenov ES, Radionov N, Berchatova AA, Popova NV, Serova OV, Petrenko AG. Structural determinants of the insulin receptor-related receptor activation by alkali. *J Biol Chem*. 2013; 288:33884–33893. [PubMed: 24121506]
7. Belfiore A, Malaguarnera R. Insulin receptor and cancer. *Endocrine-related cancer*. 2011; 18:R125–R147. [PubMed: 21606157]
8. Baserga R, Peruzzi F, Reiss K. The IGF-1 receptor in cancer biology. *International journal of cancer J Int du cancer*. 2003; 107:873–877.
9. De Meyts P, Whittaker J. Structural biology of insulin and IGF1 receptors: implications for drug design. *Nat Rev Drug Discov*. 2002; 1:769–783. [PubMed: 12360255]
10. Hubbard SR, Wei L, Ellis L, Hendrickson WA. Crystal structure of the tyrosine kinase domain of the human insulin receptor. *Nature*. 1994; 372:746–754. [PubMed: 7997262]
11. Hubbard SR. Crystal structure of the activated insulin receptor tyrosine kinase in complex with peptide substrate and ATP analog. *EMBO J*. 1997; 16:5572–5581. [PubMed: 9312016]
12. Garrett TP, McKern NM, Lou M, Frenkel MJ, Bentley JD, Lovrecz GO, Elleman TC, Cosgrove LJ, Ward CW. Crystal structure of the first three domains of the type-1 insulin-like growth factor receptor. *Nature*. 1998; 394:395–399. [PubMed: 9690478]
13. Munshi S, Kornienko M, Hall DL, Reid JC, Waxman L, Stirdivant SM, Darke PL, Kuo LC. Crystal structure of the Apo, unactivated insulin-like growth factor-1 receptor kinase. Implication for inhibitor specificity. *J Biol Chem*. 2002; 277:38797–38802. [PubMed: 12138114]
14. Munshi S, Hall DL, Kornienko M, Darke PL, Kuo LC. Structure of apo, unactivated insulin-like growth factor-1 receptor kinase at 1.5 Å resolution. *Acta Crystallogr Sect D Biol Crystallogr*. 2003; 59:1725–1730. [PubMed: 14501110]
15. McKern NM, Lawrence MC, Streltsov VA, Lou MZ, Adams TE, Lovrecz GO, Elleman TC, Richards KM, Bentley JD, Pilling PA, Hoyne PA, Cartledge KA, Pham TM, Lewis JL, Sankovich SE, Stoichevska V, Da Silva E, Robinson CP, Frenkel MJ, Sparrow LG, Fernley RT, Epa VC, Ward CW. Structure of the insulin receptor ectodomain reveals a folded-over conformation. *Nature*. 2006; 443:218–221. [PubMed: 16957736]
16. Croll TI, Smith BJ, Margetts MB, Whittaker J, Weiss MA, Ward CW, Lawrence MC. Higher-Resolution structure of the human insulin receptor ectodomain: multi-modal inclusion of the insert domain. *Structure*. 2016; 24:469–476. [PubMed: 26853939]
17. Carpenter EP, Beis K, Cameron AD, Iwata S. Overcoming the challenges of membrane protein crystallography. *Curr Opin Struct Biol*. 2008; 18:581–586. [PubMed: 18674618]
18. Pandey A, Shin K, Patterson RE, Liu XQ, Rainey JK. Current strategies for protein production and purification enabling membrane protein structural biology. *Biochem Cell Biol*. 2016; 94:507–527. [PubMed: 27010607]
19. Cuatrecasas P. Affinity chromatography and purification of the insulin receptor of liver cell membranes. *Proc Natl Acad Sci U S A*. 1972; 69:1277–1281. [PubMed: 4504339]

20. Jacobs S, Shechter Y, Bissell K, Cuatrecasas P. Purification and properties of insulin receptors from rat liver membranes. *Biochem Biophys Res Commun.* 1977; 77:981–988. [PubMed: 901526]
21. Harrison LC, Itin A. Purification of the insulin receptor from human placenta by chromatography on immobilized wheat germ lectin and receptor antibody. *J Biol Chem.* 1980; 255:12066–12072. [PubMed: 7440588]
22. Siegel TW, Ganguly S, Jacobs S, Rosen OM, Rubin CS. Purification and properties of the human placental insulin receptor. *J Biol Chem.* 1981; 256:9266–9273. [PubMed: 7021559]
23. Fujita-Yamaguchi Y, Choi S, Sakamoto Y, Itakura K. Purification of insulin receptor with full binding activity. *J Biol Chem.* 1983; 258:5045–5049. [PubMed: 6833291]
24. Roth RA, Cassell DJ. Insulin receptor: evidence that it is a protein kinase. *Science.* 1983; 219:299–301. [PubMed: 6849137]
25. Petruzzelli L, Herrera R, Rosen OM. Insulin receptor is an insulin-dependent tyrosine protein kinase: copurification of insulin-binding activity and protein kinase activity to homogeneity from human placenta. *Proc Natl Acad Sci U S A.* 1984; 81:3327–3331. [PubMed: 6203118]
26. Finn FM, Titus G, Horstman D, Hofmann K. Avidin-biotin affinity chromatography: application to the isolation of human placental insulin receptor. *Proc Natl Acad Sci U S A.* 1984; 81:7328–7332. [PubMed: 6390435]
27. Newman JD, Harrison LC. Homogeneous bivalent insulin receptor: purification using insulin coupled to 1,1'-carbonyldiimidazole activated-agarose. *Biochem Biophys Res Commun.* 1985; 132:1059–1065. [PubMed: 4074346]
28. Roth RA, Morgan DO, Beaudoin J, Sara V. Purification and characterization of the human brain insulin receptor. *J Biol Chem.* 1986; 261:3753–3757. [PubMed: 3005298]
29. Tavare JM, Denton RM. Studies on the autophosphorylation of the insulin receptor from human placenta. Analysis of the sites phosphorylated by two-dimensional peptide mapping. *Biochem J.* 1988; 252:607–615. [PubMed: 3166375]
30. Paul JJ, Tavare J, Denton RM, Steiner DF. Baculovirus-directed expression of the human insulin receptor and an insulin-binding ectodomain. *J Biol Chem.* 1990; 265:13074–13083. [PubMed: 2198283]
31. Bhaumick B, Bala RM, Hollenberg MD. Somatomedin receptor of human placenta: solubilization, photolabeling, partial purification, and comparison with insulin receptor. *Proc Natl Acad Sci U S A.* 1981; 78:4279–4283. [PubMed: 6270667]
32. Kull FC Jr, Jacobs S, Su YF, Svoboda ME, Van Wyk JJ, Cuatrecasas P. Monoclonal antibodies to receptors for insulin and somatomedin-C. *J Biol Chem.* 1983; 258:6561–6566. [PubMed: 6304046]
33. LeBon TR, Jacobs S, Cuatrecasas P, Kathuria S, Fujita-Yamaguchi Y. Purification of insulin-like growth factor I receptor from human placental membranes. *J Biol Chem.* 1986; 261:7685–7689. [PubMed: 3011795]
34. Maly P, Luthi C. Purification of the type I insulin-like growth factor receptor from human placenta. *Biochem Biophys Res Commun.* 1986; 137:695–701. [PubMed: 3015126]
35. Morgan DO, Jarnagin K, Roth RA. Purification and characterization of the receptor for insulin-like growth factor I. *Biochemistry.* 1986; 25:5560–5564. [PubMed: 2946318]
36. Blanquart C, Boute N, Lacasa D, Issad T. Monitoring the activation state of the insulin-like growth factor-I receptor and its interaction with protein tyrosine phosphatase 1B using bioluminescence resonance energy transfer. *Mol Pharmacol.* 2005; 68:885–894. [PubMed: 15976035]
37. Kavran JM, McCabe JM, Byrne PO, Connacher MK, Wang Z, Ramek A, Sarabipour S, Shan Y, Shaw DE, Hristova K, Cole PA, Leahy DJ. How IGF-1 activates its receptor. *Elife.* 2014; 3
38. Sparrow LG, Lawrence MC, Gorman JJ, Strike PM, Robinson CP, McKern NM, Ward CW. N-linked glycans of the human insulin receptor and their distribution over the crystal structure. *Proteins.* 2008; 71:426–439. [PubMed: 17957771]
39. Ullrich A, Gray A, Tam AW, Yang-Feng T, Tsubokawa M, Collins C, Henzel W, Le Bon T, Kathuria S, Chen E, et al. Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. *EMBO J.* 1986; 5:2503–2512. [PubMed: 2877871]

40. Graslund S, Nordlund P, Weigelt J, Hallberg BM, Bray J, Gileadi O, Knapp S, Oppermann U, Arrowsmith C, Hui R, Ming J, dhe-Paganon S, Park HW, Savchenko A, Yee A, Edwards A, Vincentelli R, Cambillau C, Kim R, Kim SH, Rao Z, Shi Y, Terwilliger TC, Kim CY, Hung LW, Waldo GS, Peleg Y, Albeck S, Unger T, Dym O, Prilusky J, Sussman JL, Stevens RC, Lesley SA, Wilson IA, Joachimiak A, Collart F, Dementieva I, Donnelly MI, Eschenfeldt WH, Kim Y, Stols L, Wu R, Zhou M, Burley SK, Emtage JS, Sauder JM, Thompson D, Bain K, Luz J, Gheyi T, Zhang F, Atwell S, Almo SC, Bonanno JB, Fiser A, Swaminathan S, Studier FW, Chance MR, Sali A, Acton TB, Xiao R, Zhao L, Ma LC, Hunt JF, Tong L, Cunningham K, Inouye M, Anderson S, Janjua H, Shastry R, Ho CK, Wang D, Wang H, Jiang M, Montelione GT, Stuart DI, Owens RJ, Daenke S, Schutz A, Heinemann U, Yokoyama S, Bussow K, Gunsalus KC. Structural Genomics C, China Structural Genomics C, Northeast Structural Genomics C. Protein production and purification. *Nat Methods*. 2008; 5:135–146. [PubMed: 18235434]
41. Kato H, Faria TN, Stannard B, Roberts CT Jr, LeRoith D. Role of tyrosine kinase activity in signal transduction by the insulin-like growth factor-I (IGF-I) receptor. Characterization of kinase-deficient IGF-I receptors and the action of an IGF-I-mimetic antibody (alpha IR-3). *J Biol Chem*. 1993; 268:2655–2661. [PubMed: 7679099]
42. Whittaker J, Okamoto AK, Thys R, Bell GI, Steiner DF, Hofmann CA. High-level expression of human insulin receptor cDNA in mouse NIH 3T3 cells. *Proc Natl Acad Sci U S A*. 1987; 84:5237–5241. [PubMed: 3299382]
43. Keefe AD, Wilson DS, Seelig B, Szostak JW. One-step purification of recombinant proteins using a nanomolar-affinity streptavidin-binding peptide, the SBP-Tag. *Protein Expr Purif*. 2001; 23:440–446. [PubMed: 11722181]
44. Kobayashi T, Morone N, Kashiya T, Oyamada H, Kurebayashi N, Murayama T. Engineering a novel multifunctional green fluorescent protein tag for a wide variety of protein research. *PLoS One*. 2008; 3:e3822. [PubMed: 19048102]
45. Geiser M, Cebe R, Drewello D, Schmitz R. Integration of PCR fragments at any specific site within cloning vectors without the use of restriction enzymes and DNA ligase. *Biotechniques*. 2001; 31:88–90. 92. [PubMed: 11464525]
46. Morgenstern JP, Land H. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res*. 1990; 18:3587–3596. [PubMed: 2194165]
47. Bravo DA, Gleason JB, Sanchez RI, Roth RA, Fuller RS. Accurate and efficient cleavage of the human insulin proreceptor by the human proprotein-processing protease furin. Characterization and kinetic parameters using the purified, secreted soluble protease expressed by a recombinant baculovirus. *J Biol Chem*. 1994; 269:25830–25837. [PubMed: 7929288]
48. Laprise MH, Grondin F, Dubois CM. Enhanced TGFbeta1 maturation in high five cells coinfecting with recombinant baculovirus encoding the convertase furin/pace: improved technology for the production of recombinant proproteins in insect cells. *Biotechnol Bioeng*. 1998; 58:85–91. [PubMed: 10099264]
49. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*. 2012; 9:671–675. [PubMed: 22930834]
50. Barker SC, Kassel DB, Weigl D, Huang X, Luther MA, Knight WB. Characterization of pp60c-src tyrosine kinase activities using a continuous assay: autoactivation of the enzyme is an intermolecular autophosphorylation process. *Biochemistry*. 1995; 34:14843–14851. [PubMed: 7578094]
51. Strop P, Brunger AT. Refractive index-based determination of detergent concentration and its application to the study of membrane proteins. *Protein Sci*. 2005; 14:2207–2211. [PubMed: 16046633]
52. Erickson HP. Size and shape of protein molecules at the nanometer level determined by sedimentation, gel filtration, and electron microscopy. *Biol Proced Online*. 2009; 11:32–51. [PubMed: 19495910]
53. Seddon AM, Curnow P, Booth PJ. Membrane proteins, lipids and detergents: not just a soap opera. *Biochim Biophys Acta*. 2004; 1666:105–117. [PubMed: 15519311]

54. Ellis L, Clauser E, Morgan DO, Edery M, Roth RA, Rutter WJ. Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. *Cell*. 1986; 45:721–732. [PubMed: 3518947]
55. Kato H, Faria TN, Stannard B, Roberts CT Jr, LeRoith D. Essential role of tyrosine residues 1131, 1135, and 1136 of the insulin-like growth factor-I (IGF-I) receptor in IGF-I action. *Mol Endocrinol*. 1994; 8:40–50. [PubMed: 7512194]
56. Mi LZ, Grey MJ, Nishida N, Walz T, Lu C, Springer TA. Functional and structural stability of the epidermal growth factor receptor in detergent micelles and phospholipid nanodiscs. *Biochemistry*. 2008; 47:10314–10323. [PubMed: 18771282]
57. Rothenberg PL, Lane WS, Karasik A, Backer J, White M, Kahn CR. Purification and partial sequence analysis of pp185, the major cellular substrate of the insulin receptor tyrosine kinase. *J Biol Chem*. 1991; 266:8302–8311. [PubMed: 2022647]
58. Talbot K, Wang HY, Kazi H, Han LY, Bakshi KP, Stucky A, Fuino RL, Kawaguchi KR, Samoyedny AJ, Wilson RS, Arvanitakis Z, Schneider JA, Wolf BA, Bennett DA, Trojanowski JQ, Arnold SE. Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation, and cognitive decline. *J Clin Invest*. 2012; 122:1316–1338. [PubMed: 22476197]
59. Moller DE, Yokota A, White MF, Pazianos AG, Flier JS. A naturally occurring mutation of insulin receptor alanine 1134 impairs tyrosine kinase function and is associated with dominantly inherited insulin resistance. *J Biol Chem*. 1990; 265:14979–14985. [PubMed: 2168397]
60. Casnellie JE. Assay of protein kinases using peptides with basic residues for phosphocellulose binding. *Methods Enzymol*. 1991; 200:115–120. [PubMed: 1956315]
61. Songyang Z, Carraway KL 3rd, Eck MJ, Harrison SC, Feldman RA, Mohammadi M, Schlessinger J, Hubbard SR, Smith DP, Eng C, et al. Catalytic specificity of protein-tyrosine kinases is critical for selective signalling. *Nature*. 1995; 373:536–539. [PubMed: 7845468]
62. Mulvihill MJ, Cooke A, Rosenfeld-Franklin M, Buck E, Foreman K, Landfair D, O'Connor M, Pirritt C, Sun Y, Yao Y, Arnold LD, Gibson NW, Ji QS. Discovery of OSI-906: a selective and orally efficacious dual inhibitor of the IGF-1 receptor and insulin receptor. *Future Med Chem*. 2009; 1:1153–1171. [PubMed: 21425998]
63. Pike LJ, Eakes AT, Krebs EG. Characterization of affinity-purified insulin receptor/kinase. Effects of dithiothreitol on receptor/kinase function. *J Biol Chem*. 1986; 261:3782–3789. [PubMed: 3005300]
64. Favelyukis S, Till JH, Hubbard SR, Miller WT. Structure and autoregulation of the insulin-like growth factor 1 receptor kinase. *Nat Struct Biol*. 2001; 8:1058–1063. [PubMed: 11694888]

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ab.2017.08.011>.

Fig. 1.

SDS-PAGE under reducing conditions results in separation of the $\alpha_2\beta_2$ holoreceptor into the α -subunit (α) and β -subunit (β). Receptor subunits were visualized by Western blotting using the antibodies listed under each image. Note that the SBP-tag was introduced at the C-terminus of the β -subunit, so the α -subunit is not visualized by anti-SBP-tag Western blotting. Stable HEK 293 T cells expressing IR (**A**) and IGF1R (**B**) produce high quantities of processed receptor. (**C**) Comparison of the accumulation of precursor (p) and processed β -subunit in transient vs. stable expression of IR and IGF1R.

Fig. 2. SDS-PAGE analysis of IR and IGF1R eluted from Strep-Tactin resin. Proteins were visualized with Coomassie Blue staining. Treatment with ss-furin results in cleavage of the $\alpha\beta$ precursor (p) into the corresponding α and β subunits. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Superose 6 chromatograms of IR (**A**) and IGF1R (**B**) (black) superimposed over the trace of protein standards (blue): (1) thyroglobulin, 669 kDa (2) apoferritin, 443 kDa (3) alcohol dehydrogenase, 150 kDa (4) carbonic anhydrase, 29 kDa. The eluted fractions were analyzed by Western blotting with anti-SBP-tag antibody (top), which recognizes the receptor β -subunits. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

The autophosphorylation activity of IR in various detergents with or without stimulation by insulin. Reactions were analyzed by Western blotting with anti-pYpY (to measure IR autophosphorylation) and anti-SBP-tag (to normalize protein amounts). The SBP-tag is present on the β -subunits of IR and IGF1R. Table 1 lists detergent descriptions and quantification of IR activation.

Fig. 5. Ligand-stimulated autophosphorylation of IR (**A**) or IGF1R (**B**). Autophosphorylation was measured by Western blotting with anti-pYpY antibodies, and protein levels were measured by anti-SBP-tag blotting. The SBP-tag is present on the β -subunits of the receptors.

Fig. 6. Peptide phosphorylation by IR and IGF1R. **(A)** A radiometric phosphorylation assay measuring the phosphorylation of a peptide substrate in the presence of [γ - 32 P]-ATP after 15 min at 30 °C. Some reactions included 200 nM linsitinib. **(B)** A continuous assay was used to measure rates of peptide phosphorylation for IR immediately after thawing from -80 °C, or after storage at 4 °C for 24 h.

Fig. 7. Steady state kinetic measurements of IR (**A**) and IGF1R (**B**) using a radiometric phosphorylation assay.

Table 1

Detergent screen for IR activation. The right-hand column shows IR activation derived from band intensities in Fig. 4. To calculate activation in the presence of insulin, intensities of pYpY bands were first divided by the corresponding SBP-tag band intensity. Activation was then calculated by normalizing insulin stimulated values to the unstimulated values. For example, an activation value of 1 represents equal autophosphorylation in the presence or absence of insulin.

No	Detergent	Type	CMS (mM)	IR Activation
A1	Octyl maltoside, fluorinated	Non ionic	1.02	1.78
A2	ANAPOE® NID-P40	Non ionic	0.3	1.29
A3	1,2-Diheptanoyl-sn-Glycero-3-Phosphocholine	Zwitterionic	1.4	0.90
A4	Big CHAP,deoxy	Non ionic	1.4	1.23
A5	C12E8	Non ionic	0.09	2.52
A6	ANAPOE®-C10E9	Non ionic	1.3	0.77
B1	CYCLOFO®-4	Zwitterionic	14	0.95
B2	CYGLU-3	Non ionic	28	1.59
B3	CYMAL®-6	Non ionic	0.56	1.76
B4	n-DOdecyl-β-D-maltoside	Non ionic	0.17	6.34
B5	Dimethyldodecylphosphine oxide	Non ionic	0.57	1.39
B6	FOC Choline®-10	Zwitterionic	11	0.85
C1	HECAMEG	Non ionic	19.5	1.07
C2	n-Hexyl-β-D-glucoside	Non ionic	250	1.27
C3	MEGA-10	Non ionic	7	1.13
C4	n-Decyl-α-D-maltoside	Non ionic	1.6	2.17
C5	DDMAB	Zwitterionic	4.3	0.92
C6	n-Nonyl-β-D-glucoside	Non ionic	6.5	1.02
D1	n-Octyl-β-D-glucoside	Non ionic	18	0.79
D2	CHAPS	Zwitterionic	8	1.53
D3	n-Undecyl-N,N-Dimethylamine-Oxide	Zwitterionic	3.2	0.99
D4	TWEEN® 80	Non ionic	0.01	1.18
D5	n-Decanoylsucrose	Non ionic	2.5	1.72
D6	ZWITTERGENT® 3-08	Zwitterionic	330	1.22

Table 2

Kinetic constants were calculated by fitting radiometric phosphorylation assay data (Fig. 7) to a non-linear curve using the Michaelis-Menten equation. The bottom part of the table gives previously reported values for kinetic parameters. IGF1R-KD consists of the isolated kinase domain of the IGF1R receptor.

	K_m (μ M)	V_{max}	Specific Activity (nmol/min/mg)	Substrate
IR	235 ± 26	32.08 ± 0.95	171	KKEEEEYMMMMG
IGF1R	386 ± 109	65.92 ± 6.28	354	KKEEEEYMMMMG
IR [25]	–	–	80	Angiotensin
IR [63]	–	–	117	RRLIEDAEYAARG
IGF1R [37]	66	–	–	KKEEEEYMMMMG
IGF1R-KD [64]	1300	–	2100	KKEEEEYMMMMG