



Cross-species reactive monoclonal antibodies against the extracellular domains of the insulin receptor and IGF1 receptor

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ABSTRACT

Translation across species of immunoassay results is often challenging due to the lack of cross-species reactivity of antibodies. In order to investigate the biology of insulin and IGF1 receptors, we generated new versatile monoclonal assay antibodies using the extracellular domain of the insulin/IGF1 hybrid receptor as the bait protein in the Adimab yeast antibody discovery platform and as the antigen in a rabbit monoclonal antibody platform. The resulting antibody clones were screened for receptor specificity as well as cross-species reactivity to both tissue and cell line derived samples. Using these strategies, we were able to identify highly specific insulin receptor monoclonal antibodies that lack cross-reactivity to the IGF1 receptor using the Adimab platform and a highly specific IGF1 receptor monoclonal antibody that lacks cross-reactivity to the insulin receptor using the rabbit antibody platform. Unlike earlier monoclonal antibodies reported in the literature, these antibodies show cross-species reactivity to the extracellular domains of mouse, rat, pig, and human receptors, indicating that they bind conserved epitopes. Furthermore, the antibodies work well in several different assay formats, including ELISA, flow cytometry, and immunoprecipitation, and therefore provide new tools to study insulin and IGF1 receptor biology with translation across several species and experimental model systems.

1. Introduction

Type 1 diabetes, which most frequently occurs in juveniles, is an autoimmune disease that leads to specific eradication of insulin-producing beta cells. In most cases, type 2 diabetes is linked to obesity-associated insulin resistance, which eventually progresses into hyperglycaemia. Although the molecular biology of type 2 diabetes has been studied intensively for many decades, the underlying molecular aetiology of the disease is not well understood. Type 2 diabetes is a polygenetic disease that seems to be caused by a number of different deleterious modalities that all lead to insulin resistance, which complicates translation from animal models to human disease. Even though mammalian biochemistry is largely similar across species, there are also many species differences that provide pitfalls for translation, especially between commonly used inbred rodent models and human diseases (Chandrasekera and Pippin, 2014).

The insulin receptor (IR) is at the centre stage of glucose homeostasis. Cross-species data on IR expression and activation are crucial for improving our understanding of the molecular aspects of disease and for the development of new and improved treatment options. Along with the growing number of obese persons, the number of new type 2 diabetes patients has reached epidemic proportions over the last three decades, and both academia and the pharmaceutical industry are searching for an enhanced understanding of the disease as well as new ways to improve treatments and ultimately finding a cure or prevention (Levien and Baker, 2009). There are still challenges in disease management, as many patients exhibit poor glycaemic control and experience hypoglycaemic events, weight gain, late complications and comorbidities (Pantalone et al., 2015; American Diabetes Association, 2018). To improve the next generation of treatments, the field requires facilitated translation of insulin biology between experimental animals and humans, as well as between in vitro and in vivo data, which is

Abbreviations: WT, wild type; mAb, monoclonal antibody; pAb, polyclonal antibody; PBS, phosphate buffered saline

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dependent on sensitive assays that are easily translated across species.

The IR and IGF1 receptor (IGF1R) are closely related tetrameric receptor tyrosine kinases, with their ectodomains sharing 53% sequence identity (Xu et al., 2018). They both consist of two alpha-subunits responsible for ligand binding and two kinase-containing beta-subunits. The beta-subunit is mostly intracellular and linked to the extracellular alpha subunits by a single transmembrane region and a disulphide bridge located in a relatively short, protruding extracellular domain of the beta-subunit. The alpha/beta subunits are derived from a single larger precursor that is cleaved inside the Golgi compartment (Forsayeth et al., 1986). IR and IGF1R belong to a subset of receptor tyrosine kinase family members in which the two alpha-subunits are connected by preformed disulphide bridges leading to dimeric receptor complexes in the absence of ligand. The suggested role of the alpha subunits in the unbound state is to keep the two trans-phosphorylating beta subunits apart. Ligand binding induces conformational changes that bring the two kinase domains closer, allowing autophosphorylation and downstream signaling to occur (Lemmon and Schlessinger, 2010; Kavran et al., 2014; Gutmann et al., 2018; Scapin et al., 2018).

IR plays an important role in metabolic regulation, whereas IGF1R is thought to mainly play a role in growth and tissue homeostasis. IR and IGF1R have an approximately 100-fold preference for their intrinsic ligands. Signaling by IR is complicated by the effects of the differential splicing of exon 11, which encodes 12 amino acids in the C-terminal part of the IR alpha subunit generating two IR isoforms termed IR-A and IR-B (IR-B containing the additional 12 amino acids). The biological role of the two isoforms is not fully understood, but interestingly, IR-A has a 10-fold higher affinity for both IGF1 and IGF2 compared to IR-B, with IGF2 at a level leaving it within physiologically-relevant levels (Frasca et al., 1999; Andersen et al., 2017). The ratio between the two isoforms of IR varies among different tissues. IR-A is highly expressed in human kidneys and the brain, while IR-B is the predominant isoform found in the human liver (Møller et al., 1989; Sesti et al., 1994). Additionally, IR can also form hybrid receptors with IGF1R, leading to six different possible combinations of functional dimers with different ligand affinities. IR/IGF1R hybrid receptors can be found in most tissues, and the recent development of IR/IGF1R hybrid assay technology may shed light on the largely unknown biological function of the hybrid receptor (Slaaby et al., 2006; Slaaby, 2015).

Existing IR and IGF1R antibodies often generate conflicting results due to a lack of cross-species reactivity. To generate new IR and IGF1R antibodies that can be used in cross-species translational experiments, we used the Adimab platform and a rabbit monoclonal antibody platform, together with the ectodomain of the IR/IGF1R hybrid receptor as bait protein or antigen, respectively. Additionally, we used a combined screening of the resulting antibody clones on targets from both in vitro and in vivo derived mouse, rat, pig and human samples.

2. Materials and methods

2.1. IR/IGF1R ectodomain antigen/bait protein

Soluble human IR/IGF1R hybrid receptor ectodomain was prepared using stable co-expression in BHK cells of the N-terminally FLAG tagged IR ectodomain (tagged after the first five amino acids: HLYPG-DYKDDDDK-) and C-terminally haemagglutinin (HA) tagged IGF1R ectodomain (See Fig. 6), both constructs being inserted into the pZem expression vector (Andersen et al., 1992). The IR/IGF1R hybrid receptor ectodomain was purified using an Anti-FLAG M2 affinity resin (Sigma-Aldrich) and synthetic FLAG peptides for elution, followed by a gel-filtration step (S300 sephacryl gel, GE healthcare). Various attempt to purify the resulting protein further using an anti-HA agarose resin was unsuccessful, indicating that the C-terminal HA tag was not accessible in the folded conformation of the protein. Therefore, the protein preparation was most likely a mixture of hybrid receptors and IR homodimers.

2.2. Adimab mAbs

We used the fully-human naïve Adimab yeast antibody discovery platform to select antibodies against the IR/IGF1R hybrid receptor. A preparation of human ectodomain IR/IGF1R hybrid receptors was randomly biotinylated, labelled with phycoerythrin (PE) conjugated streptavidin and used as bait protein for the selection of specific antibody-expressing yeast clones. Eight separate libraries representing different antibody families each with a $1-2 \times 10^9$ diversity were screened for specific binders through consecutive rounds of enrichment using magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS). Briefly, two rounds of MACS using a bait protein concentration of 100 nM were performed before three rounds of FACS, including a de-selection round for depletion of poly-specific binders. After the final selection round, clones were plated, and 95 clones were picked and sequenced from each of the eight libraries. We identified 428 unique antibodies from the pool of sequences, and these were expressed and purified for further analysis.

2.3. Rabbit/human chimeric mAbs

All experimental procedures involving animals were performed under a license granted by the national Danish authority, The Animal Experiment Inspectorate. Rabbits were immunised four times with the IR/IGF1R hybrid receptor ectodomain antigen. All immunisations were performed subcutaneously using 50 µg of antigen in Freund's Adjuvant. Blood was drawn ten days after the last immunization and used for sera tests. Splenic B cells were harvested from positive rabbits and single-cell sorted via a FACS AriaII (BD Biosciences) using FITC-labelled anti-rabbit IgG (Jackson ImmunoResearch) and biotinylated IR/IGF1R ectodomain labelled with Streptavidin-PE (Jackson ImmunoResearch). Sytox red (Invitrogen) was included as a dead live stain. Sorted cells were expanded in vitro for 10 days on a layer of irradiated EL4-B5 cells, and the culture supernatant was harvested and used for ELISA screening. ELISA-positive clones were cloned as described in Lightwood et al., 2006. In short, the heavy/light chain variable regions were cloned using RT-PCR with gene specific primers and the amplicons inserted into an expression vector containing human IgG1/kappa constant regions. The construct was expressed in HEK cells, and the specificity of the chimeric rabbit/human antibody was validated by ELISA and flow cytometry screens using BHK cells that overexpressed IR or IGF1R.

2.4. Generation of HCT116 IGF1R and IR gene knockout cells

Transcription activator-like effector nucleases (TALEN) fragments IGF1R-TAL-L, IGF1R-TAL-R, IR-TAL-L and IR-TAL-R were cloned into the Gateway pcDNA-DEST40 vector (Thermo Fisher Scientific) to generate TALEN plasmids silencing the expression of either human IGF1R or IR. HCT116 colon carcinoma cells (5×10^5) were transfected with TALEN plasmids targeting human IGF1R or human IR encoding genes using Lipofectamine LTX Reagent (ThermoFisher) as described by the manufacturer. Five days after transfection, the cells were trypsinised and stained using either 24–31 hIGF1R mouse mAb or 83–7 hIR mouse mAb (licensed from K. Siddle, University of Cambridge, UK), followed by staining with a PE-F(ab')₂ Goat anti-mouse IgG (H + L) (Jackson ImmunoResearch). IGF1R- and IR-negative cells were sorted as single cells using FACS and were then clonally expanded in McCoy's 5A culture medium (ATCC) supplemented with 1% penicillin-streptomycin (P/S), (Gibco) and 10% heat inactivated foetal calf serum (FBS, Gibco) for ~10–15 days. 24 surviving clones were analysed for receptor expression by FACS. The IGF1R- or IR-negative clones were further characterized using genomic PCR amplification and sequencing (Taihegene, China). Clones with double-allelic IGF1R or IR knockouts were selected and named HCT116-IGF1RKO and HCT116-IRKO, respectively.

2.5. Phosphospecific sandwich ELISA

HCT116-IGF1RKO and HCT116-IRKO cells were cultured in McCoy 5A cell culture medium. Before stimulation, the cells were serum starved for 60 min in McCoy 5A culture medium containing 0.1% human serum albumin (Sigma). The cells were then stimulated for 30 min with 200 nM human insulin or human IGF1 diluted in starvation medium. Thereafter the cells were washed briefly in ice-cold PBS, and cell extraction buffer (Invitrogen) was added that contained 1 mM AEBSF proteinase inhibitor (Calbiochem) and 1 mM proteinase inhibitor cocktail (Sigma). The protein concentration was measured using a BCA Protein Assay kit according to the manufacturer's description (Pierce). Greiner Microtron Bio-One ELISA plates (Greiner) were coated with 2 µg/ml Affinipure F(ab')₂ goat anti-human IgG Fc (Jackson ImmunoResearch) in carbonate coating buffer, pH 9.6, overnight and then washed four times in Tris buffer saline with 0.05% Tween 20 (TBS-T tablets, Sigma) and then incubated in blocking buffer for 1 h (StartingBlock T20 blocking buffer, Thermo Fisher Scientific). Adimab antibody clone supernatants were diluted 1:10 in PBS containing 0.5 M NaCl and 0.5% Tween-20 and incubated for 1 h. The plates were washed four times in TBS-T, and protein extracts from HCT116 cells were added at a concentration of 0.5 mg/ml total protein and incubated for 1.5 h at 37 °C. Thereafter, the plates were washed four times in TBS-T, and mouse 4G10 anti-phosphotyrosine mouse mAb (Merck) diluted in blocking buffer was added and the plates were incubated for 1.5 h at 37 °C. Next, the plates were washed four times in TBS-T, and horse radish peroxidase (HRP) conjugated donkey anti-mouse IgG (H + L) (Jackson ImmunoResearch) diluted in blocking buffer added and incubated for 30 min at RT. The plates were washed four times; finally, TMB substrate (tetramethylbenzidine, Sigma) was added and incubated for ~45 min, and plates were read on Spectramax photospectrometre at 450 nm and the signal was plotted in relative units (RU).

2.6. Quantification of surface IR and IGF1R

Human IR or IGF1R receptors expressed on the surface of cells grown to 80% confluence in McCoy's 5A medium with 10% FBS were quantified using the QIFIKIT kit (Dako) according to the manufacturer's protocols. Briefly, cell cultures were detached using TrypLE Express (Gibco) and were then centrifuged and resuspended in PBS. Cell suspensions were stained with either the 83–7 hIR mouse mAb, 24–31 hIGF1R mouse mAb, or an isotype control antibody. Cells were analysed with flow cytometry using an LSRFortessa instrument (BD Biosciences).

2.7. Flow Cytometry

HCT116-WT, HCT116-IGF1RKO, and HCT116-IRKO cells were detached with TrypLE Express (Gibco) and incubated for 30 min with either 227, D2, G6, or human IgG isotype control mAb each diluted to 0.4 nM. The cells were washed three times in FACS buffer and stained with APC conjugated donkey anti-human IgG (Jackson ImmunoResearch) and washed additionally three times in FACS buffer before being analysed using LSRFortessa and Kaluza software.

2.8. Immunoprecipitation and western blot analysis of HCT116 cells

Analysis of the mAb specificity by immunoprecipitation of protein extracts from HCT116-WT, HCT116-IRKO, and HCT116-IGF1RKO cells using Dynabeads and western blot analyses was performed as follows: IR mAbs D2 and G6 and IGF1R mAb 227 were coupled to magnetic Dynabeads M270 Epoxy (Thermo Fisher Scientific) as described by the manufacturers, and the antibody-coated beads were stored in PBS at pH 7.4 containing 0.1% Tween-20 and 0.02 sodium azide until they were used. 0.5 mg antibody conjugated beads was washed once in PBS and resuspended in 1 mg of total protein extracts from HCT116-WT,

HCT116-IRKO, or HCT116-IGF1RKO cells, and incubated for 1 h at RT with gentle rocking. The beads were washed three times in PBS and resuspended in 130 µl of ddH₂O, 50 µl of loading buffer (NuPage) and 20 µl of a reducing agent (NuPage) and heated to 97 °C for seven minutes. The beads were removed, and the immunoprecipitated samples were submitted to standard gel electrophoresis followed by western blotting. Insulin Rβ (C-19) SC-711 rabbit pAb (Santa Cruz Biotechnology) was used for blotting of IR and D23H3 rabbit mAb (Cell Signaling Technology) was used for blotting IGF1R. Horse radish peroxidase (HRP)-conjugated goat anti-rabbit (Jackson ImmunoResearch) was added, and bands were detected by Western Bright Quantum Chemoluminescence (Advanta).

2.9. IR and IGF1R detection in tissue

Frozen samples of liver and muscle tissues from mice, rats and pigs were homogenized using a mortar and pestle cooled with dry ice. The samples were lysed for four minutes at speed 30 in Cell Extraction Buffer (Invitrogen) using a TissueLyser II (Qiagen). The homogenates were cleared by 15 min of centrifugation at 5000 x g at 4 °C. The supernatants were combined at a 1:1 ratio with buffer that consisted of 100 mM NaCl, 10 mM MgSO₄, 100 mM HEPES and 0.025% Tween-20, pH 7.8. To remove unspecific bead binding, 10% v/v protein G-coupled agarose beads (Roche) were added and incubated for one hour at 4 °C while they were slowly rotated and were then centrifuged at 5000 x g and 4 °C for three minutes; the pellet was then discarded. The protein content was determined using a BCA Protein Assay Kit (Pierce) following the instructions of the manufacturer. An equal protein concentration was used in 1 ml of the above-mentioned buffer. 60 µl of G-protein coupled to agarose beads and 1 mg of antibody (D2, G6, 227, 24–31 or 83–7) was added and incubated overnight rotating at 4 °C. Samples were centrifuged and washed three times with ice-cold TBS-T. The IR specific mAb 83–7 and IGF1R specific mAb 24–31 were licensed from K. Siddle, University of Cambridge, UK. Immunoprecipitates were released from G-protein coupled agarose beads by adding a Bond-Breaker TCEP Solution (Thermo Fisher Scientific), XT Sample Buffer 4 × (BioRad) and incubated for five minutes at 95 °C. The samples were then analysed by standard western blotting using the anti-IR C-19 (Santa Cruz) and anti-IGF1R N-20 (Santa Cruz) antibodies as primary antibodies for standard Western blotting procedure. For Western blot analysis of BHK cells over-expressing IR or IGF1R with D2, G6, or 227 mAbs the secondary antibody was anti-human-HRP (Jackson ImmunoResearch).

3. Results

3.1. Adimab antibodies

We used the naïve Adimab human IgG1 yeast discovery platform to isolate monoclonal antibodies (mAbs) against conserved IR or IGF1R epitopes. An ectodomain preparation of the human IR/IGF1R hybrid receptor was used as bait protein for the clone selection.

Following several rounds of enrichment for specific binders, 760 clones were sequenced. From these 428 unique antibody clones that bound to the bait protein were identified, expressed, and purified for further binding analysis by flow cytometry using BHK cells over-expressing full length human IR (220,000 hIR/cell) or human IGF1R (760,000 hIGF1R/cell). This process led to identification of 126 mAbs binding BHK-hIR cells with mean fluorescence intensity at least 10 fold over that of a BHK mock and 10 low-affinity mAbs specific for hIGF1R (not shown). The 126 hIR specific binders represented 14 of the 19 VH germlines present in the version of the Adimab library used here.

HCT116 colon carcinoma cells (HCT116-WT) endogenously express 5100 IR and 2800 IGF1R/cell, which is comparable to the endogenous level of expression found in most cell lines. To study the binding specificity, we generated protein extracts from human HCT116 IR gene

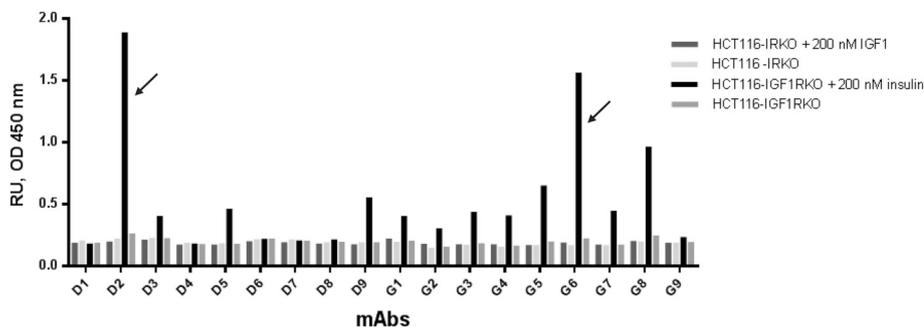


Fig. 1. Representative figure showing examples of Adimab mAb screening using receptor activation-specific sandwich ELISA. IGF1R was activated in HCT116-IRKO (IGF1R only) by the addition of 200 nM IGF1, and IR was activated in HCT116-IGF1RKO (IR only) by the addition of 200 nM human insulin. Two IR mAbs, D2 and G6, were selected (marked by arrows). No Adimab clones specific for IGF1R were detected.

knockout cells (HCT116-IRKO) that expressed 6700 IGF1R/cell and HCT116 IGF1R gene knockout cells (HCT116-IGF1RKO) that had 3100 IR/cell. To obtain semi-quantitative data, ELISA plates were coated with a polyclonal goat anti-human capture antibody (pAb), and exposed to excess antibody supernatant from the different clones to ensure a comparable total amount of mAbs in each well. In this experiment, the mAb clones function as receptor capture antibodies. To activate IR and IGF1R receptors, HCT116-IRKO (IGF1R only) cells were stimulated with 200 nM IGF1 and HCT116-IGF1RKO (IR only) with 200 nM insulin, leading to tyrosine phosphorylation of the respective beta-subunits. To detect tyrosine phosphorylation in the beta-subunit (receptor activation), a 4G10 anti-phosphotyrosine mouse mAb was added, followed by the additions of an anti-mouse HRP conjugated donkey pAb, and the resulting signal was measured using an ELISA reader. > 30 clones were selected as positive (> 3-fold over the unstimulated control). Furthermore, no cross-reaction was observed to HCT116-IRKO cells, and the signal was only observed after ligand-stimulated receptor phosphorylation demonstrated specificity in a functional assay. The two best performing IR mAbs, designated D2 and G6, were selected for further evaluation (Fig. 1). No antibodies specific for IGF1R were selected.

3.2. Monoclonal rabbit antibodies

In order to increase our panel of anti-IGF1R mAbs, we used a rabbit B-cell platform. Following immunization of rabbits with the IR/IGF1R ectodomain and primary B-cell selection, a total of 49 anti-IGF1R specific clones were chosen for cloning and recombinant expression in a human IgG1 scaffold. Binding of the expressed rabbit/human chimeric mAbs was confirmed by ELISA and flow cytometry using BHK cells that overexpressed IGF1R as described above for the Adimab mAbs. Additionally, mAb specificities were validated via immunoprecipitation of purified receptors from BHK cells that overexpressed human IR or IGF1R, and rat IGF1R (not shown). IGF1R mAb 227 were selected for further analysis.

3.3. Specific immunoprecipitation of IR and IGF1R by selected mAbs

The anti-IR mAbs D2 and G6, and anti-IGF1R mAb 227 were further tested for specificity by immunoprecipitation using protein extracts from HCT116-WT, HCT116-IRKO and HCT116-IGF1RKO. In the following western blotting IR was detected by commercial beta-subunit specific C-19 rabbit pAb and IGF1R was detected by commercial beta-subunit specific D23H3 rabbit mAb (Fig. 2 A-F). D2 and G6 mAbs were shown to selectively immunoprecipitate IR since IR was immunoprecipitated from HCT116-IGFRKO and HCT116-WT and not from HCT116-IRKO (Fig. 2 A, C). The D2 and G6 IR mAbs also immunoprecipitated IGF1R from HCT116-WT cells because of the presence of endogenous IR/IGF1R hybrid receptors, where the IR mAbs co-immunoprecipitate IGF1R linked to IR in the hybrid molecule (Fig. 2 B, D). The 227 antibody selectively immunoprecipitated IGF1R since IGF1R was immunoprecipitated from HCT116-IRKO and HCT116-WT



Fig. 2. Western blotting analysis of IR versus IGF1R specificity by immunoprecipitation (IP) performed using protein extracts from HCT116-WT, HCT116-IRKO (IGF1R only) and HCT116-IGF1RKO (IR only) cells. D2 and G6 successfully immunoprecipitated IR from HCT116-WT and HCT116-IGF1RKO (IR only), as detected using a C-19 IR C-terminal specific blotting antibody (IB) (A and C). D2 and G6 also immunoprecipitated the hybrid receptor from HCT116-WT, as detected using a D23H3 IGF1R C-terminal specific antibody (IB) (B and D). 227 immunoprecipitated IGF1R from HCT116-WT and HCT116-IRKO (IGF1R only), as detected using a D23H3 IGF1R C-terminal specific blotting antibody (IB) (E). 227 also immunoprecipitated the hybrid receptor, as detected using a C-19 IR C-terminal specific blotting antibody (IB) (F). The formation of IR/IGF1R hybrid receptors was exclusively observed in HCT116-WT cells that expressed both receptors (B, D, and F).

and not from HCT116-IGF1RKO (Fig. 2 E). Likewise, 227 also co-immunoprecipitated IR/IGF1R hybrid receptors from HCT116-WT, where both IR and IGF1R are expressed leading to the formation of IR/IGF1R hybrid receptors (Fig. 2 F). Importantly, the IR/IGF1R hybrid receptor was immunoprecipitated exclusively from HCT116-WT cells and was not found when either IR or IGF1R were genetically removed. Thus, these mAbs in combination with beta-subunit specific IR or IGF1R antibodies may provide new tools to study the largely unknown biology of IR/IGF1R hybrid receptors. The IR mAbs D2 and G6 and IGF1R mAb 227 show no cross-reactivity and are specific for their respective targets.

3.4. Analysis of holoreceptor binding by flow cytometry

In order to test whether the selected antibodies were able to detect native IR and IGF1R on the cell surface, HCT116-WT, HCT116-IRKO and HCT116-IGF1RKO cells were stained with 227, D2 and G6 and

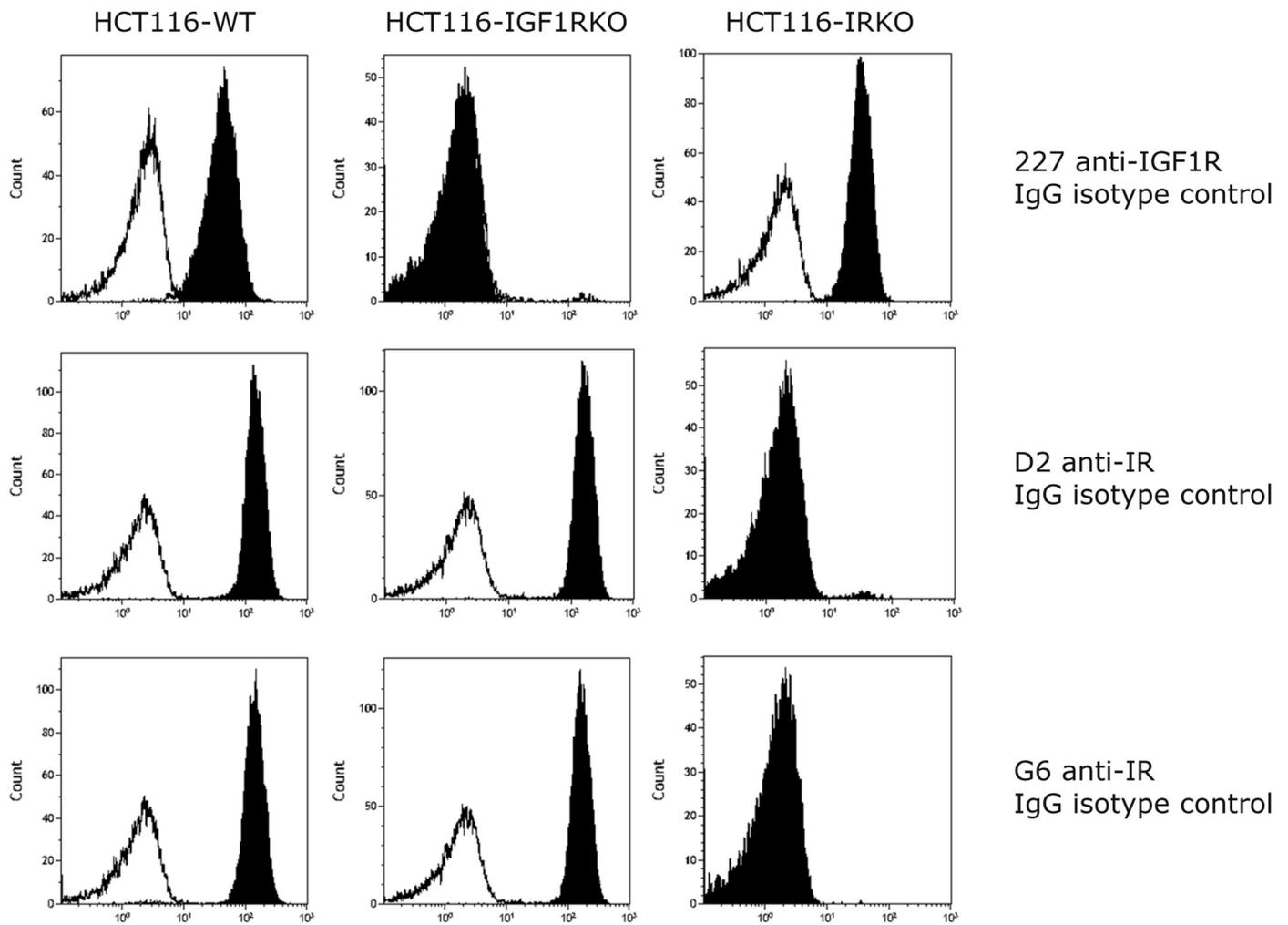


Fig. 3. FACS analysis of the cell surface binding and specificity of the 227, D2 and G6 mAbs. In the upper panels, 227 anti-IGF1R binding (black) is compared to an isotype control mouse IgG1 (white). Binding to HCT116-WT and HCT116-IRKO is demonstrated, whereas no binding is observed in HCT116-IGF1RKO cells. In the middle and lower panels, binding of D2 and G6 anti-IR to HCT116-WT and HCT116-IGF1RKO is observed, whereas no binding to HCT116-IRKO is observed. These results demonstrate the specificity of the mAbs for binding to the native membrane-bound receptor conformations at the cell surface.

analysed using FACS. In this experiment, expression of IGF1R by 227 was readily detected on HCT116-WT and HCT116-IRKO and not HCT116-IGF1RKO. D2 and G6 readily detected IR on HCT116-WT and HCT116-IGF1RKO, but not on HCT116-IRKO. This shows specificity of 227, D2, and G6 mAbs towards their respective cell surface holoreceptor antigens (Fig. 3).

3.5. Cross-species reactivity of anti-IR and anti-IGF1R antibodies

Target binding across species were tested by immunoprecipitation of liver and muscle tissue preparations from mice, rats and pigs. To test IR-specific mAbs, livers were homogenized, and the resulting protein homogenates underwent immunoprecipitation using the IR antibodies D2, G6 and the commonly used 83–7 IR ectodomain-specific mouse mAb (Fig. 4 upper panel) (Soos et al., 1986). The D2 and G6 antibodies immunoprecipitated IR from protein homogenates isolated from mice, rats and pigs, whereas 83–7 immunoprecipitate homogenates from pig, but failed to immunoprecipitate IR in protein samples from rats and mice. To test IGF1R mAbs, muscle tissues from mice, rats, and pigs were homogenized, and protein isolates from these tissues underwent immunoprecipitation with IGF1R mAb 227 and the widely used 24–31 IGF1R ectodomain-specific mouse mAb (Soos et al., 1992). The IGF1R antibody 227 immunoprecipitated IGF1R from muscle tissue isolates from mice, rats and pigs, in contrast to 24–31 (Fig. 4 lower panel). This

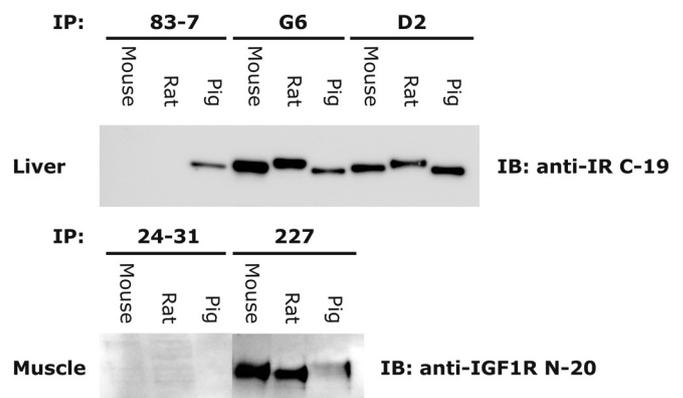


Fig. 4. Immunoprecipitation (IP) of muscle and liver samples using commercially available anti-IR 83–7 and anti-IGF1R 24–31 mAbs compared to the newly generated anti-IR G6 and D2 mAbs and anti-IGF1R 227 mAb. C-terminal specific anti-IR C-19 and anti-IGF1R N-20 were used to detect immunoprecipitated proteins through immunoblotting (IB). The antibody 83–7 shows ability to immunoprecipitate IR from pig but not mouse and rat muscle samples, whereas D2 and G6 readily immunoprecipitate IR from samples from mice, rats and pigs. The antibody 24–31 does not immunoprecipitate IGF1R from any of the tested sample, whereas 227 show cross-species immunoprecipitation of samples from mice, rats, and pigs.

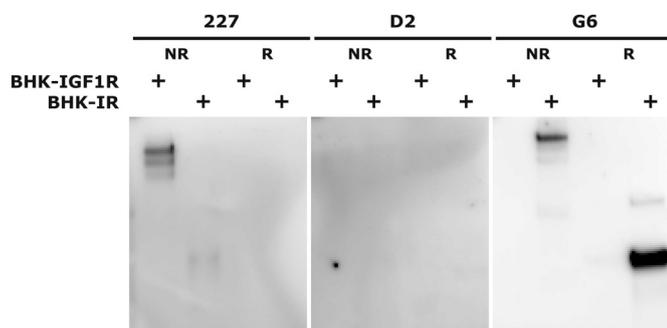


Fig. 5. Western blot analysis of human IGF1R or IR from overexpressing BHK cells. Protein samples were WGA purified and analysed in Western blotting under non-reducing (NR) and reducing (R) conditions. Anti-IGF1R 227 exclusively bound to IGF1R under non-reducing conditions. Anti-IR D2 mAb did not bind to IR in Western blotting and therefore most likely is sensitive to small conformational changes induced by the experimental procedure. Anti-IR G6 mAb bound IR under both non-reducing and reducing conditions showing that binding was not sensitive to a specific IR conformations.

experiment shows cross-species reactivity to tissue samples from mouse, rat and pig (other species not tested), indicating that these new mAbs are important new tools in translational diabetes research.

3.6. Antibody binding in western blotting

To characterize antibody/epitope interaction the antibodies were analysed in western blotting under non-reducing and reducing condition. Non-denaturing condition largely preserves the antigen conformation and disulphide bridges, whereas reducing condition breaks disulphide bridges, denature protein, and induces linearisation of epitopes. The anti-IGF1R 227 mAb exclusively bound to IGF1R under non-reducing conditions, indicating binding dependent on a conformational epitope. The anti-IR D2 mAb did not show any binding after gel-electrophoresis, although D2 works perfectly well in ELISA and FACS and most likely some minor conformational changes prevent D2 binding. In contrast, anti-IR G6 mAb bound both to non-reduced and reduced IR indicating binding to a linear IR epitope. No cross-reaction of IGF1R mAbs to IR and conversely no binding of IR mAbs to IGF1R were seen (Fig. 5).

4. Discussion

In this study, we characterize newly generated antibodies that can provide further translational tools for the investigation of two closely

related insulin and IGF1 receptors. We used the human IR/IGF1R hybrid ectodomain, as a “dual” antigen/bait protein that contains the extracellular portion of both IR and IGF1R in a functional heterodimeric conformation. IR and IGF1R share a high degree of sequence and structural homology; hence, the specificity of mAbs for IR or IGF1R is essential in order to build specific assays.

Ligand-induced IR and IGF1R activation are commonly measured using antibody-based sandwich assays that include the specific capture of the relevant ectodomain of either IR or IGF1R or, alternatively, capture the C-terminal end of the intracellular domain, which is followed by detection of tyrosine specific phosphorylation in the intracellular kinase domain. While the phosphotyrosine consensus sites in the tyrosine kinase activation loop as well as their flanking amino acids are generally conserved between species and phospho-specific antibodies raised to this region, therefore, show cross-species reactivity, most of the current commercial alpha-subunit capture antibodies show poor cross-species reactivity and binding to tissue-derived IR and IGF1R from rodents. (Fig. 4, Table 1). Using classic mouse hybridoma technology, it is often difficult to generate monoclonal antibodies against epitopes that are conserved between the mouse and other species. Therefore, mouse monoclonal antibodies, such as mouse IR mAb 83-7 and mouse IGF1R mAb 24-31, do not bind mouse and rat targets, which limits their use in cross-species translation experiments using rodents, although they work perfectly well with human samples (Soos et al., 1986; Soos et al., 1992). Rabbits seem to show a larger diversity in their antibody repertoire compared to mice, and they readily generate antibodies that are cross-reactive to rodent and human epitopes (Knight and Winstead, 1997; Weber et al., 2017). The Adimab yeast antibody discovery platform contains a naïve antibody library suitable for generating antibodies to structural epitopes that are conserved between species and thus ideal to target IR and IGF1R epitopes that are conserved between species (Sivasubramanian et al., 2017).

In this study, the selection strategy used with the Adimab system seemed to be biased towards generating antibodies against IR, with little generation of IGF1R antibodies. However, the rabbit mAb platform generated antibodies against IGF1R that demonstrated a comparably higher affinity. Furthermore, only a sub-fraction of the IR antibody clones showed binding to tissue-derived IR (data not shown), and this is most likely explained by a more pronounced tissue-specific glycosylation that masks certain epitopes.

D2 and 227 mAbs show poor binding to denatured proteins in Western blotting, indicating that they bind structural epitopes, whereas G6 not seems to be dependent on a conformational epitope. A search for IR/IGF1R hybrid-specific antibodies was included in the screen, but no antibodies fulfilled the criteria for hybrid specific binding. Based on

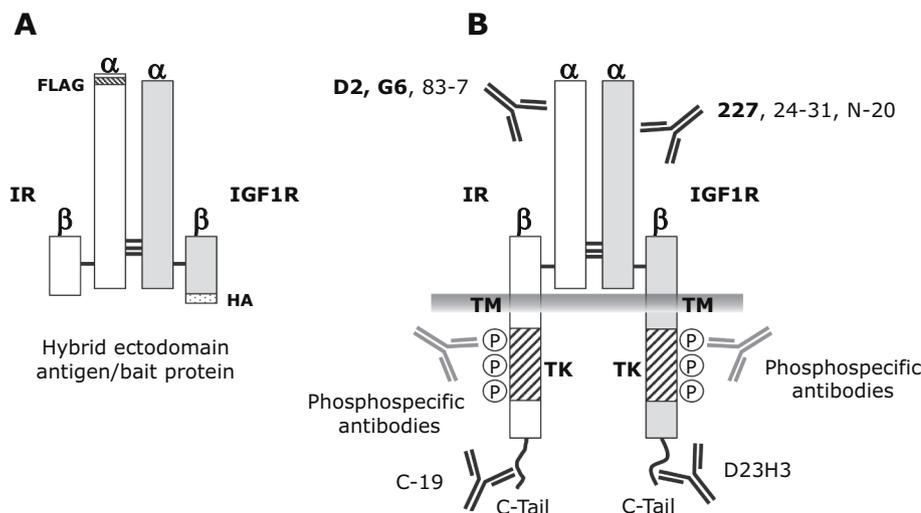


Fig. 6. Schematic overview showing the ectodomain IR/IGF1R hybrid receptor antigen/bait protein with FLAG and HA tags used for tandem purification (A) and the schematic holoreceptor structure with markings of where the different antibodies binds (B). The transmembrane spanning domain is indicated as TM. TK outlines the tyrosine kinase activation domain (hatched). New antibodies are marked in bold.

Table 1
Species reactivity and overview of the IR and IGF1R assay antibodies used in this study.

Name	Specificity	Reactivity	Type	Source
D2	IR alpha-subunit	H, M, R, P	Human IgG1 mAb	In-house
G6	IR alpha-subunit	H, M, R, P	Human IgG1 mAb	In-house
227	IGF1R alpha-subunit	H, M, R, P	Rabbit/human chimeric IgG1 mAb	In-house
83-7	IR alpha-subunit	H, B, P, Rab	Mouse IgG1 mAb	In-house ^a
24-31	IGF1R alpha-subunit	H	Mouse IgG1 mAb	In-house ^a
D23H3	IGF1R beta-subunit	H, M, R, Mk	Rabbit IgG mAb	Cell Signaling Technology
C-19 (SC711)	IR C-terminus	H, M, R, P	Rabbit pAb	Santa Cruz Biotechnology
N-20 (SC712)	IGF1R N-terminus	H, M, R, P	Rabbit pAb	Santa Cruz Biotechnology
4G10 Platinum	Phosphotyrosine	Pan specific	Mouse IgG2b cocktail	Merck

^a Produced under license from Dr. K. Siddle, University of Cambridge, UK and are also commercial available. Human (H), mouse (M), rat, (R), pig (P), Bovine (B), Rabbit (Rab) and monkey (Mk).

their sensitivity in endpoint assays and human/rodent assay translatability, we finally selected two IR mAb clones (D2 and G6) that were derived using the Adimab platform and an IGF1R mAb clone (227) that were derived using the rabbit mAb platform.

The inclusion of different animal model species and human cell lines for antibody selection led to the identification of antibodies that can be used in studies of IR and IGF1R that can be translated across species in a semi-quantitative manner. The 227 antibody is the first monoclonal IGF1R ectodomain-specific antibodies that demonstrate cross-species reactivity to mouse, rat, pig, and human tissues and exhibit qualities that allow translation between in vitro and in vivo derived samples. Similarly, D2 and G6 are highly specific antibodies to IR that also allow cross-species translation in both in vitro and vivo systems. Here, we have showed that these antibodies can be used in immunoprecipitations, FACS and ELISA assays as well as western blotting for anti-IR G6. In addition, D2 was recently shown to be useful in phospho-specific sandwich ELISA that detects IR activation in rat tissue samples after treatment with different doses of insulin (Hvid et al., 2018). The antibodies described here provide new important tools that bridge some of the translational gaps between especially rodent and human insulin and IGF1 biology.

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Disclosure of potential conflicts of interest

All authors, except Laura Hvidsten Ørstrup, at the time of performing the work in this manuscript were employees of Novo Nordisk A/S and therefore have potential conflict of interest in being employed by the organisation that could potentially benefit from the publication of this work. The study was fully funded by Novo Nordisk.

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