A Novel Insulin Analog With Unique Properties

Lys^{B3},Glu^{B29} Insulin Induces Prominent Activation of Insulin Receptor Substrate 2, but Marginal Phosphorylation of Insulin Receptor Substrate 1

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The potentially enhanced mitogenic activity of insulin analogs represents a safety risk that requires detailed analysis of new analogs considered for therapeutic applications. We assessed the signaling properties and mitogenic potency of two novel rapid-acting insulin analogs, Lys^{B3},Glu^{B29} insulin (HMR 1964) and Lys^{B3},Ile^{B28} insulin (HMR 1153) using myoblasts and cardiomyocytes. In myoblasts, both binding and internalization were two- to threefold higher for Asp^{B10} insulin and HMR 1153 when compared with HMR 1964 and regular insulin. This finding correlated with a prominent Shc/IGF-I receptor interaction, tyrosine phosphorylation of Shc, activation of extracellular signal−regulated protein kinase (ERK)-1 and -2, and stimulation of DNA synthesis by HMR 1153 and Asp^{B10} insulin. In contrast, HMR 1964 produced a marginal activation of the Shc/ERK kinase cascade and was equipotent to insulin in stimulating DNA synthesis in myoblasts. Further, the in vivo growth-promoting activity of this analog was found to be identical to that of regular human insulin. In myoblasts, HMR 1964 produced a minor activation of insulin receptor substrate (IRS)-1 tyrosine phosphorylation, but a prominent activation of IRS-2, with a significantly stronger effect than insulin in human myoblasts. Predominant activation of IRS-2 was also observed in adult cardiomyocytes where HMR 1964 increased 3-*O*-methylglucose transport and the activation of Akt and glycogen synthase kinase-3 to the same extent as human insulin. We concluded that *1*) the mitogenic properties of insulin analogs may result from a series of initial receptor interactions, including internalization and phosphorylation; *2*) the mitogenic and metabolic potential of HMR 1964 is identical to that of insulin; and *3*) predominant activation of IRS-2 may open new avenues for optimized insulin therapies. *Diabetes* 52:2227–2238, 2003

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This article is dedicated to Prof. Dr. Hans Reinauer on the occasion of his 70th birthday.

BrdU, bromo-2′-deoxyuridine; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; ERK, extracellular signal−regulated protein kinase; GSK-3, glycogen synthase kinase-3; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; TCA, trichloroacetic acid.

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Insulin therapy of diabetic patients aims to achieve tight blood glucose control to reduce the progression of long-term complications (1). However, the pharmacokinetic characteristics of currently available insulin preparations are unable to mimic the pattern of endogenous insulin secretion and make it impossible to achieve sustained normoglycemia (2). Great efforts have been made to develop novel insulin molecules with altered pharmacodynamic characteristics that might lead to improved glycemic control using recombinant DNA technology (rev. in 3–5). One limiting factor is the slow absorption of conventional unmodified insulin from subcutaneous tissues because of the slow dissociation rate of hexameric insulin complexes into monomers at the injection site (6,7). Modification of the B26−B30 region of the insulin molecule, particularly substitution of amino acids with charged residues at the association sites, allows the production of a range of insulin analogs with reduced self-association that exhibit no profound perturbations of insulin receptor recognition (4,8). This has been demonstrated for insulin analogs such as Lispro (Lys^{B28},Pro^{B29}) insulin and insulin aspart (Asp^{B28} insulin), two rapid-acting insulins that are in clinical use and improve postprandial glycemic control (3,5).

A major concern related to the long-term use of insulin analogs stems from the observation that modification of the insulin molecule in the B10 and B26−B30 regions alters the affinity for the IGF-I receptor more than for the insulin receptor and may lead to enhanced mitogenic activity of these analogs (9). This potential safety risk was first recognized for the analog Asp^{B10} insulin, which was found to exhibit a tumor-promoting activity in SD rats (10) and to induce a profound mitogenic effect in many cell systems (11–13). The enhanced mitogenic signaling profile of an insulin analog may result from *1*) an increased affinity toward the IGF-I receptor, resulting in augmented IGF-I receptor signaling (9); *2*) the so-called time-dependent specificity that describes a distinct correlation between the mitogenic potential and the occupancy time at the insulin receptor for a given insulin analog (14); and *3*) a combination of both IGF-I and insulin receptor−mediated processes. Most recent data suggest that the mitogenic properties correlate better with the IGF-I recep-

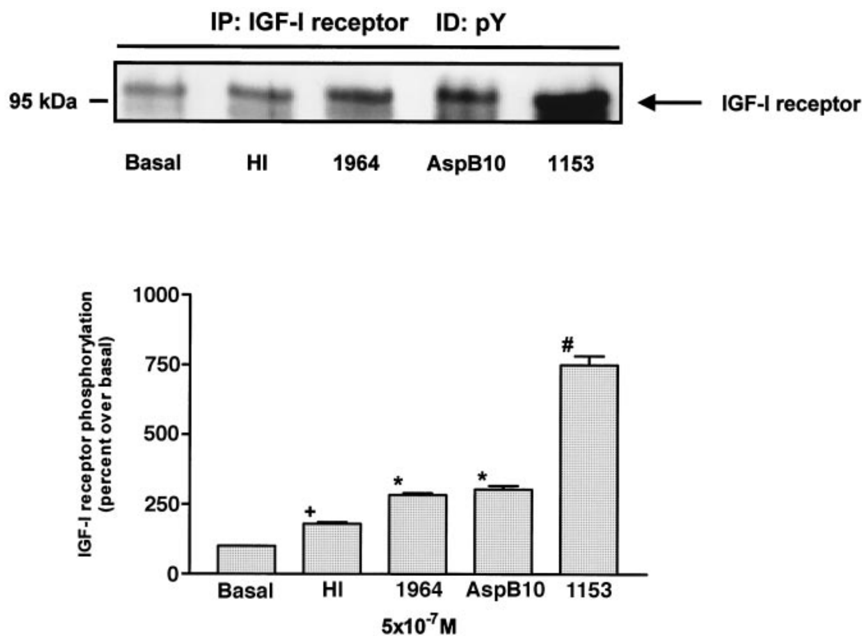

FIG. 1. Autophosphorylation of the IGF-I receptor in K6 myoblasts in response to insulin and insulin analogs. Myoblasts were stimulated for 10 min with human insulin (HI) or the indicated analogs at a concentration of 500 nmol/l, and the IGF-I receptor was immunoprecipitated (IP) as described. The immunopellet was analyzed by SDS-PAGE, blotted, and immunodetected (ID) with anti-phosphotyrosine (pY) antibodies using the ECL system. Quantification was performed on a LumiImager work station. Results are expressed relative to the basal value and are means ± SE of three separate experiments. *P < 0.006 vs. human insulin; #P < 0.001 vs. human insulin; +P < 0.05 vs. all other values.

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tor affinities than with the insulin receptor off-rates (12). The increased mitogenic potency and potential carcinogenic effect of prolonged exposure to high dosages of Asp^{B10} insulin has been consistently shown to result, at least in part, from the stimulation of the IGF-I receptor (15).

In the present investigation, we analyzed the signaling properties of two novel rapid-acting insulin analogs, Lys^{B3},Glu^{B29} insulin (HMR 1964) and Lys^{B3},Ile^{B28} insulin (HMR 1153) in comparison with native human insulin and the analog Asp^{B10} insulin using rat and human myoblasts and differentiated muscle cells. We attempted to correlate the mitogenic potential of the analogs to *1*) the initial receptor binding and processing, *2*) the activation of the Shc/mitogen-activated protein kinase (MAPK) pathway, and *3*) the induction of the tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and 2. The resulting data clearly show that HMR 1964 and 1153 produce highly divergent signaling patterns independent of their binding affinities for the IGF-I receptor. In an important development, we demonstrated that the mitogenic potential of HMR 1964 is identical to that of regular human insulin, and that it has the unique property of predominantly activating the IRS-2 pathway in both myoblasts and differentiated muscle cells. We therefore suggest that receptor phosphorylation and/or processing must be considered as an additional determinant of signaling specificity of the insulin molecule.

RESEARCH DESIGN AND METHODS

Materials. Native human insulin and the insulin analogs Lys^{B3},Glu^{B29} insulin (HMR 1964), Lys^{B3},Ile^{B28}(HMR 1153), and Asp^{B10} insulin as well as the ^{125}I-labeled insulin preparations (specific activity 260 mCi/mg) were provided by Aventis Pharma (Frankfurt, Germany). 3-*O*-[^{14}C]methyl-D-glucose and L-[1-^{14}C]glucose were purchased from Amersham Pharmacia Biotech (Freiburg, Germany). Reagents for SDS-PAGE were supplied by Amersham Pharmacia Biotech and Sigma (Deisenhofen, Germany). Collagenase was from Serva (Heidelberg, Germany), and BSA (Fraction V, fatty acid free) was obtained from Boehringer (Mannheim, Germany). Protein A-trisacryl (GF-2000) and protein G-agarose were products from Pierce (Oud Beijerland, the Netherlands). The monoclonal IGF-I receptor antibody was purchased from Oncogene (Cambridge, MA). The polyclonal anti-Shc, anti-IRS-1, and anti-IRS-2 antibodies were obtained from Biomol (Hamburg, Germany). IRS-1 and IRS-2 antisera used for immunoprecipitation were kindly provided by Dr. J.A. Maassen (Leiden, the Netherlands). The phosphospecific p42/44 MAPK antisera (Thr202/Tyr204), the anti-phospho-Akt (Ser473), the anti−phospho−glycogen synthase kinase (GSK)-3α and -3β (Ser21/9), and the p44/42 MAPK, anti-Akt, and anti-GSK-3 antibodies were products of New England Biolabs (Schwalbach/Taunus, Germany). The monoclonal mouse anti-rat Ki-67 antibody was obtained from Dako Cytomation (Glostrup, Denmark). The anti-phosphotyrosine antiserum RC20 was produced by Becton Dickinson (Heidelberg, Germany). Horseradish peroxidase conjugate (anti-rabbit IgG) as the secondary antibody for enhanced chemiluminescence (ECL) was purchased from Promega (Mannheim, Germany). Stripping solution was a product of Alpha Diagnostics (San Antonio, TX). The cell proliferation enzyme-linked immunosorbent assay chemiluminescence kit was purchased from Boehringer. FCS, Dulbecco's modified Eagle's medium (DMEM), nonessential amino acids, and penicillin/streptomycin were provided by Gibco (Eggenstein, Germany). Primary human skeletal muscle cells, basal medium, and the supplement pack for growth medium were obtained from PromoCell (Heidelberg, Germany). All other chemicals were of the highest grade commercially available.

Cell culture and isolation of cardiomyocytes. K6 myoblasts represented a rat heart muscle cell line that was established and characterized in our laboratory (16). These cells are insulin sensitive and express GLUT4 (16). Cells were kept in monolayer culture in DMEM supplemented with 10% FCS, nonessential amino acids (1%), streptomycin (100 μg/ml), and penicillin (100 units/ml) in 175-cm^2 flasks in an atmosphere of 5% CO_2 at 37°C. Myoblasts were maintained in continuous passages by trypsinization of subconfluent cultures 7 days after they were plated. The medium was changed every 72 h. Cell number was determined after cell dissociation with trypsin/EDTA at 37°C.

TABLE 1
Binding, internalization, and degradation of insulin and insulin analogs in rat myoblasts

	Binding (fmol/2 × 10^5 cells)	Internalization (cpm × 10^2)	Degradation (%)
Human insulin	2.8 ± 0.06	2,200 ± 200	6.3 ± 0.5†
HMR 1964	2.2 ± 0.1	1,600 ± 300	3.0 ± 0.4
AspB10	4.2 ± 0.05*	3,400 ± 400*	2.6 ± 0.7
HMR 1153	4.4 ± 0.01*	5,500 ± 1,100*	5.0 ± 0.3†

Data are means ± SE taken from 4–5 separate experiments. Binding, internalization, and degradation of insulin and insulin analogs was determined as described in RESEARCH DESIGN AND METHODS. *Significantly different from human insulin and HMR 1964 at $P < 0.05$; †significantly different from HMR 1964 and AspB10 at $P < 0.05$.

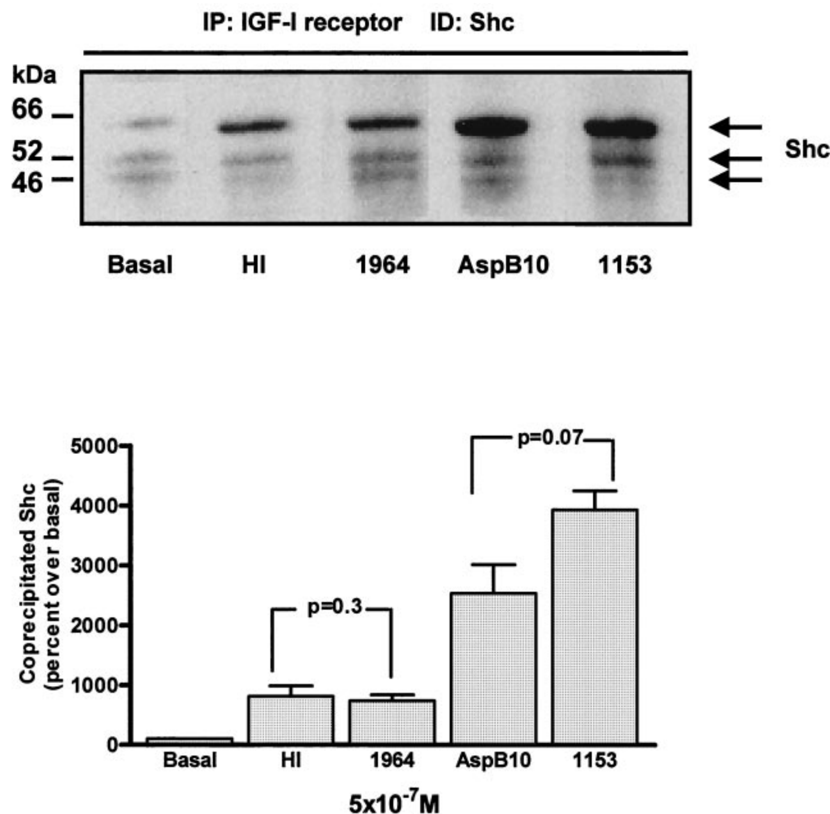

FIG. 2. Co-precipitation of Shc proteins with the IGF-I receptor after stimulation of K6 myoblasts with insulin or insulin analogs. Myoblasts were stimulated with human insulin (HI) or an analog and the IGF-I receptor was immunoprecipitated (IP), as described in Fig. 1. Immunopellets were processed and immunoblotted with anti-Shc antibodies. Quantification of the 66-kDa protein band was performed using the LumiImager system and is presented in the lower panel. Data are means ± SE of 3 separate experiments. ID, immunodetection.

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Primary human skeletal muscle cells obtained from satellite cells isolated from *M. rectus abdominis* of a 28-year-old male Caucasian donor were supplied as proliferating myoblasts. These cells were kept in a skeletal muscle cell growth medium (basal medium containing FCS, 5%; epidermal growth factor, 10 ng/ml; basic fibroblast growth factor, 1 ng/ml; fetuin, 0.5 mg/ml; insulin, 0.1 mg/ml; dexamethasone, 0.4 μg/ml; gentamicin, 50 μg/ml; and amphotericin B, 50 ng/ml) for two population doublings. Cells were then frozen and stored in liquid nitrogen for further use. For stimulation experiments, 10^6 cells/dish were seeded in growth medium and cultured for 2 days. Cells were then washed with PBS and cultured for 4 days in the absence of serum and insulin. The cells were then cultured for 1 h with fresh medium containing 0.5% BSA and subsequently stimulated with the hormones.

Adult rat cardiomyocytes were isolated by perfusion of the heart with collagenase, as previously described (17). Male Wistar rats (280–340 g) were used in all experiments. The final cell suspension was incubated for 60 min until further use in HEPES buffer (130 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 25 mmol/l HEPES, 5 mmol/l glucose, 2% [wt/vol] BSA [pH 7.4], equilibrated with oxygen) containing $MgSO_4$ and $CaCl_2$ (final concentration of 1 mmol/l) at 37°C in a rotating waterbath shaker. The cell viability was judged by determining the percentage of rod-shaped cells and averaged 90–97% under all incubation conditions.

Binding, internalization, and degradation. For binding studies, myoblasts were suspended in DMEM containing 10% FCS and seeded in six-well culture dishes at a density of 2×10^5 cells/well. After 24 h in culture, the cells were washed two times with PBS and incubated for 60 min at 37°C in DMEM without FCS containing 2% BSA. ^{125}I-labeled human insulin or one of the ^{125}I-labeled insulin analogs (0.1 μCi, 5×10^{-11} mol/l) was then added along with the corresponding unlabeled peptide hormone (10^{-8} mol/l), and incubation was continued for 10 min at 37°C. The medium was then removed, the cells were washed twice and lysed with 0.1% SDS, and radioactivity was determined in a gamma counter. Nonspecific binding was measured in parallel incubations performed in the presence of an excess of the corresponding unlabeled hormone (10^{-5} mol/l). All assays were performed in triplicate. Internalization was determined after incubation of myoblasts for 60 min at 37°C using a concentration of 5×10^{-11} mol/l (0.1 μCi) of the different insulin molecules. Unbound insulin was removed by first washing the cells with cold PBS and then washing them three times with cold PBS at acid pH (pH 2.75; 0.1% BSA). Cells were lysed in 1% SDS/0.1 N NaOH, and the remaining radioactivity was determined in a gamma counter. The same incubation conditions were used for determining the degradation of the insulin molecules. Briefly, after 60 min, aliquots of the supernatant were subjected to trichloroacetic acid (TCA) precipitation and the degradation was calculated from the increase in TCA solubility of the tracer, as outlined earlier (18).

Immunoprecipitation. K6 myoblasts (3×10^6) were plated in their regular growth medium. After a 24-h culture period, the medium was removed and replaced with fresh medium without FCS containing 0.5% BSA. After a 2-h incubation at 37°C, cells were stimulated with human insulin or one of the insulin analogs (final concentration of 5×10^{-7} mol/l) for 10 min. After being washed twice with ice-cold PBS, cells were incubated in radioimmunoprecipitation buffer (50 mmol/l Tris-HCL [pH 7.4]; 1% nonidet P-40; 0.25% Na-deoxycholate; 150 mmol/l NaCl; 1 mmol/l EDTA; 1 mmol/l phenylmethylsulfonyl fluoride; 1 μg/ml each aprotinin, leupeptin, and pepstatin; 1 mmol/l Na_3VO_4; and 1 mmol/l NaF) for 2 h at 4°C with gentle agitation. Human myoblasts were cultured as outlined above and stimulated with the insulin molecules for 10 min, followed by lysis with radioimmunoprecipitation assay buffer. Freshly isolated cardiomyocytes were preincubated in a rotating waterbath shaker according to our protocols (19); after a 10-min stimulation with the different insulin analogs, the lysis was performed using radioimmunoprecipitation buffer. For immunoprecipitation, cell lysate samples were incubated with antibodies against the IGF-I receptor, IRS-1, IRS-2, or Shc at 4°C and gently rocked overnight. The immunocomplexes were adsorbed onto protein G-sepharose or protein A-sepharose beads for 2 h at 4°C during gentle agitation before being collected by centrifugation at 14,000 rpm for 30 s. Beads were washed three times with ice-cold PBS and used for Western blot analysis.

Immunoblotting. The immunoadsorbed proteins were solubilized in Laemmli sample buffer and resolved by SDS/PAGE on 8–18% (wt/vol) horizontal gradient gels, then transferred to polyvinylidene difluoride membranes. These were then blocked in Tris-buffered saline/Tween 0.05% plus 1% BSA for 1 h at room temperature and incubated with the appropriate primary antibody at 4°C overnight. After being extensively washed, membranes were incubated with horseradish peroxidase–conjugated secondary antibodies. Protein bands were then visualized by the ECL method on a LumiImager work station. MAPK activation was assessed by immunoblotting K6 cell lysates with phosphospecific extracellular signal–regulated protein kinase (ERK)-1/2 antibodies, and the phosphorylated proteins were detected by ECL. Activation of Akt and GSK-3 in adult cardiomyocytes was determined by immunoblotting lysates with phosphospecific antibodies. Blots were stripped and reprobed with ERK-1/2, Akt, and GSK-3 antibodies, respectively. All blots were quantified using the LumiImager software.

In vitro and in vivo analysis of growth-promoting activity. To monitor DNA synthesis, K6 myoblasts (1×10^4 cells/well) were seeded in 24-well

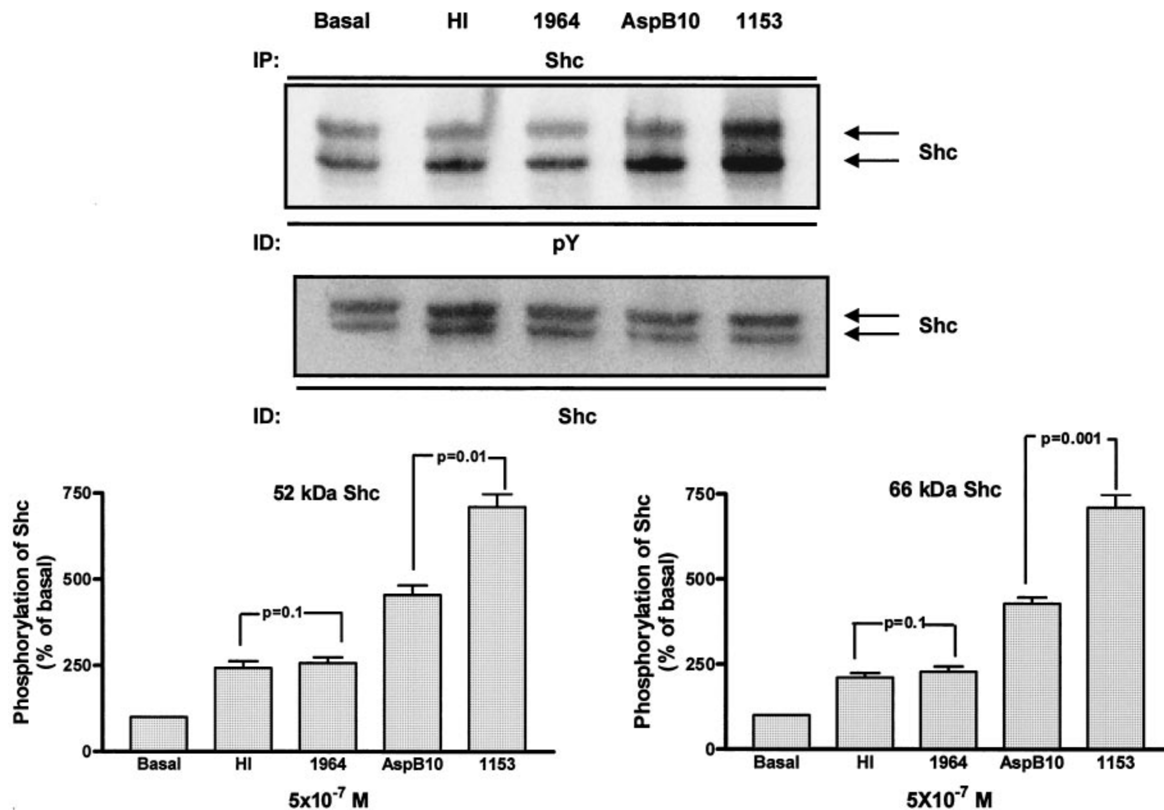

FIG. 3. Tyrosine phosphorylation of Shc proteins in K6 myoblasts in response to insulin and insulin analogs. Cells were stimulated with the peptide hormones and the Shc proteins were immunoprecipitated (IP) as described. Immunopellets were processed and immunoblotted with anti-phosphotyrosine (pY) antibodies, as outlined in Fig. 1. Equal loading was ensured by reprobing the stripped filters with anti-Shc antibodies. The 52- and 66-kDa Shc protein band was quantified using the LumiImager system. Data are means ± SE of 5 separate experiments. HI, human insulin; ID, immunodetection.

microtiter tissue culture plates and cultured for 24 h in DMEM containing 10% FCS, then cultured for 30 h under serum-free conditions. Cells were then stimulated with the different peptide hormones or 10% FCS for 16 h with the simultaneous addition of 5-bromo-2′-deoxyuridine (BrdU). After the labeling medium was removed, the cells were fixed and the DNA was denatured by adding FixDenat (Boehringer Mannheim, Germany). Cells were then incubated with a peroxidase-conjugated anti-BrdU antiserum and, after the substrate was added, the light emission was quantified on a LumiImager work station.

To assess the mitogenic effect of HMR 1964 in comparison with human insulin in vivo, a 12-month toxicity study was performed. Groups of 30 female SD rats (Charles River, Sulzfeld, Germany) were injected subcutaneously with 20 and 50 IU HMR 1964/kg, twice per day for a 12-month period. For comparison, HR 1799, a biosynthetic human regular insulin, was included at 20 IU/kg, twice per day. The control groups were treated with placebo solution. Rats were assigned to subgroups according to their stage of estrus cycle. The cycle stage was determined by examination of vaginal histology. Analysis of cell proliferation marker Ki-67 by immunohistochemistry was performed on mammary gland tissue specimens of all rats. Because of its large size, the mammary gland was divided into four tissue slices. All specimens were processed using standard protocols. After being processed for antigen retrieval, sections were incubated overnight at 4°C with a monoclonal mouse anti-rat Ki-67 antibody and then with a biotinylated rabbit anti-mouse antibody. Signals were then visualized using a streptavidin-peroxidase complex and the appropriate substrate/chromogen mix. Microphotographs were taken from randomly chosen fields; the evaluating investigators were blinded to the experimental condition. The labeling index indicative of proliferation was expressed for every rat as the percent of positive labeled nuclei per total number of counted nuclei (at least 1,000).

Determination of 3-*O*-methylglucose transport. The determination of 3-*O*-methylglucose transport using freshly isolated adult rat cardiomyocytes was performed at 37°C in HEPES buffer (130 mmol/l NaCl, 4.8 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 25 mmol/l HEPES, 5 mmol/l glucose, 20 g/l BSA [pH 7.4]). In all, 4×10^5 cells/ml were stimulated with insulin or insulin analogs for 10 min. The transport reaction was started by pipetting a 50-μl aliquot of the cell suspension to 50 μl of HEPES buffer containing 3-*O*-[^{14}C]methyl-D-glucose (final concentration of 100 μmol/l). Carrier-mediated glucose transport was then determined using a 10-s assay period and L-[^{14}C]glucose to correct for simple diffusion, as described in earlier reports from this laboratory (19,20).

Statistical analysis. All results are expressed as means ± SE, unless otherwise indicated. The significance of reported differences was evaluated using the null hypothesis and *t* statistics for paired data in all in vitro experiments. For the in vivo study, Wilcoxon's two-sample *Z* test (Mann-Whitney *U* test) was used. Corresponding significance levels are indicated in the figures.

RESULTS

Binding and processing of insulin and insulin analogs by K6 myoblasts. It has been reported that modifications in the COOH-terminal region of the B chain of insulin alter the affinity for the IGF-I receptor more than for the insulin receptor (21). As shown in our earlier studies (13), cardiac myoblasts express a high level of IGF-I receptors, with a marginal abundance of insulin receptors, and thus represent a suitable tool for assessing IGF-I receptor signaling by insulin analogs. As shown in Table 1, the analog HMR 1153 exhibited the highest binding to K6 myoblasts, comparable with that of the analog Asp^{B10} insulin, which has a reported higher affinity for the IGF-I receptors than regular insulin (22). In contrast, the analog HMR 1964 showed a significantly lower binding potency that was similar to that of human insulin. It should be noted that these binding studies were performed at a high concentration of the peptide hormones (10^{-8} mol/l) to allow comparison with the studies on signal transduction and DNA synthesis described below. One interesting finding was that HMR

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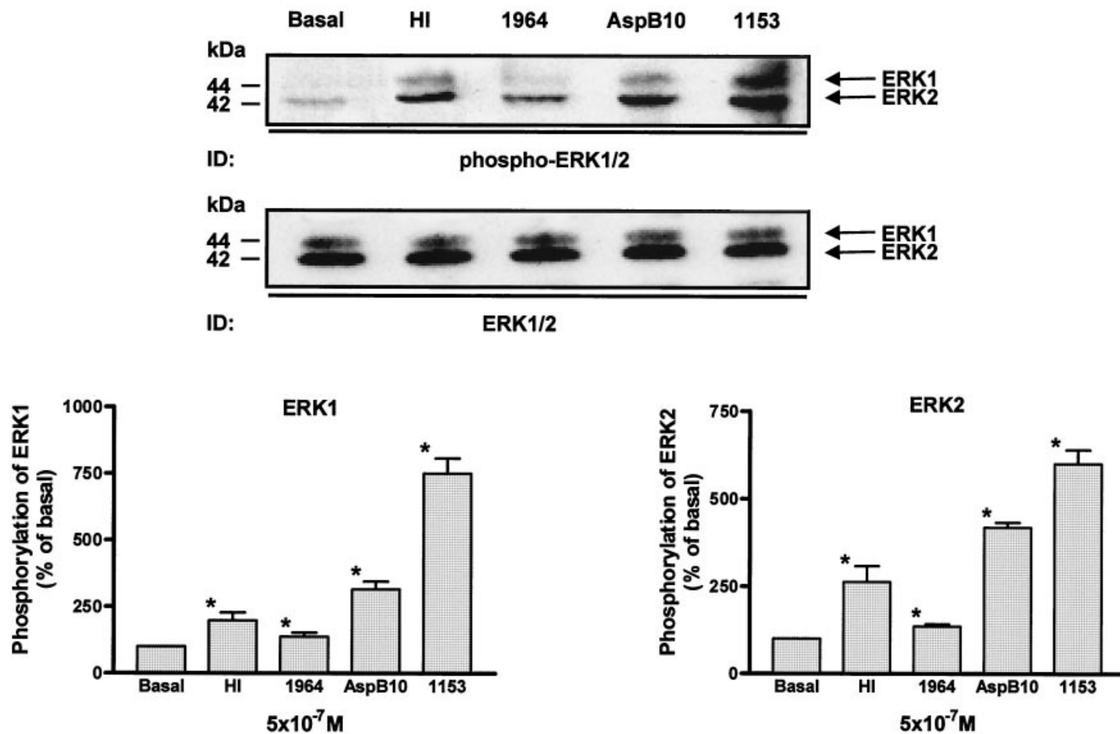

FIG. 4. Activation of p42/44 MAPK by insulin and insulin analogs in K6 myoblasts. Cells were stimulated with the different insulins as described in Fig. 1 and lysed. Cellular proteins were separated by SDS-PAGE and immunoblotted with phospho-ERK-1/2 antibodies, stripped, and reprobed with ERK-1/2 antibodies using ECL detection. Phospho-ERK-1/2 signals were quantified using the LumiImager software. Data are means ± SE obtained from 4 separate experiments. HI, human insulin; ID, immunodetection. *Significantly different from basal and all other stimulated values with at least $P < 0.05$.

1964 exhibited the lowest rate of internalization and also low degradation by the myoblasts (Table 1). The data in Table 1 show that internalization and degradation of an insulin molecule are not always directly correlated. Thus, human insulin showed a significantly lower rate of internalization compared with HMR 1153 but showed the highest degradation by K6 cells. This observation fits the notion that insulin processing is a complex process involving internalization and degradation at different intracellular sites (23).

Effect of insulin and insulin analogs on the Shc/MAPK pathway. To assess the signaling potency of the different insulin analogs to the MAPK pathway, we determined the autophosphorylation of the IGF-I receptor, the phosphorylation of Shc proteins, and the activation of p44/42 (ERK-1/2) MAPK. As presented in Fig. 1, the analog HMR 1153 induced a prominent autophosphorylation of the IGF-I receptor in the K6 myoblasts. This effect was about 2.5-fold higher than the autophosphorylation induced by Asp^{B10} insulin, despite a comparable binding of these analogs (Table 1). On the other hand, the insulin analog HMR 1964 produced the same effect as Asp^{B10} insulin, despite having the lowest binding affinity to the K6 myoblasts. This result strongly suggests that the level of receptor occupancy is not sufficient to determine the signaling potency of an insulin molecule.

This view is further supported by the data presented in Fig. 2. Immunoprecipitates of the IGF-I receptor were immunoblotted with an anti-Shc antibody that recognizes all three Shc isoforms. These adaptor proteins play a central role in the activation of the MAPK cascade (24). As can be seen from the data (Fig. 2), the 66-kDa Shc exhibited the most prominent association with the autophosphorylated IGF-I receptor in response to the insulin molecules. Asp^{B10} insulin and HMR 1153 induced a comparable association of the 66-kDa Shc with the IGF-I receptor, which was about three- to fourfold higher compared with that induced by insulin and HMR 1964. Most importantly, no significant difference was observed between human insulin and HMR 1964 at this level of the signaling cascade. The difference observed between Asp^{B10} insulin and HMR 1964, which both produce comparable autophosphorylation of the IGF-I receptor, may have resulted from the twofold higher internalization of Asp^{B10} insulin. Consistent with this finding, signaling via Shc was shown to be dependent on IGF-I receptor internalization (24). It should be noted that the much higher autophosphorylation of the IGF-I receptor by HMR 1153 did not lead to an appropriately strong interaction with Shc (Fig. 2). For all experiments, equal loading was ensured by reprobing the blots with an anti−IGF-I receptor antibody (data not shown).

To further investigate the interaction between the IGF-I receptor and the Shc proteins, tyrosine phosphorylation of these intracellular substrates was determined after stimulation of K6 myoblasts with human insulin or the insulin analogs. For this assay, the cell extracts were subjected to immunoprecipitation with an anti-Shc antibody and the resulting precipitates were analyzed by being immunoblotted with an anti-phosphotyrosine antiserum. As shown in Fig. 3, the two insulin analogs HMR 1153 and Asp^{B10} insulin induced a strong tyrosine phosphorylation of the 66- and 52-kDa Shc isoforms. Again, the analog HMR 1964 was comparable with human insulin in inducing a much

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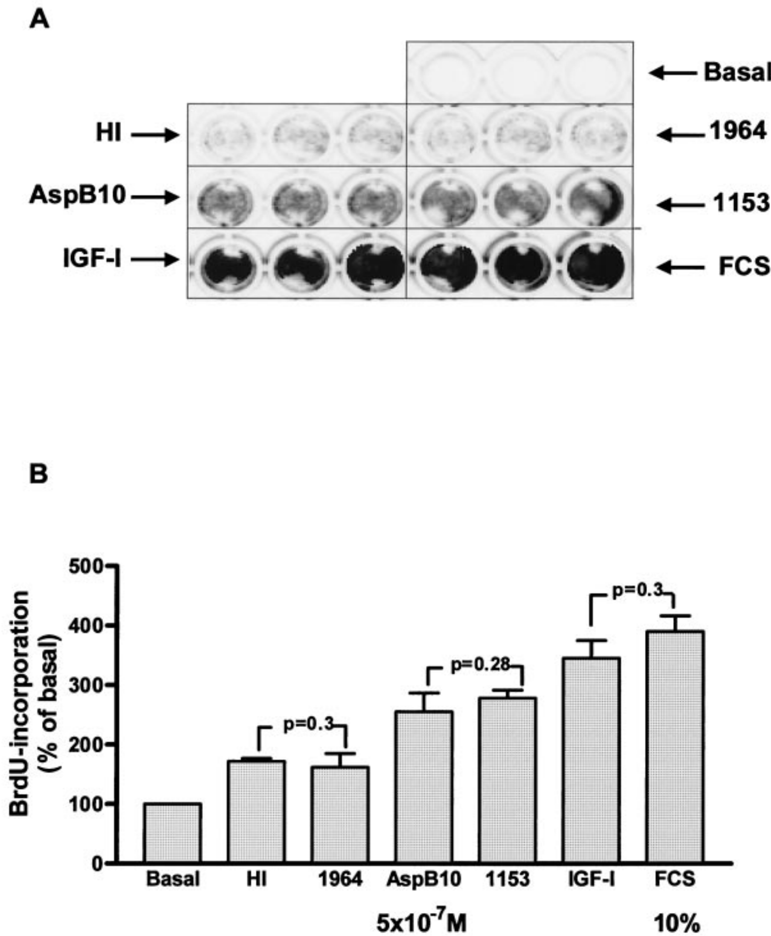

FIG. 5. Effects of insulin, insulin analogs, and IGF-I on the incorporation of BrdU into DNA in K6 myoblasts. Myoblasts were serum-starved for 30 h in DMEM and subsequently incubated with BrdU in the absence (Basal) or presence of the indicated concentrations of peptide hormones or FCS for 16 h. Cells were fixed and denatured and the incorporation of BrdU was determined using an anti-BrdU antiserum and ECL detection. *A*: LumiImager Signals obtained from triplicate cell culture wells of a representative experiment are shown. *B*: Signals were quantified using LumiImager software. Data are means ± SE of 4 separate experiments. HI, human insulin.

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lower Shc phosphorylation (Fig. 3). Quantification of tyrosine phosphorylation of the most prominent 52-kDa Shc isoform from multiple experiments demonstrated a sevenfold increase in the level of Shc phosphorylation after stimulation with HMR 1153 and a fivefold response after treatment with Asp^{B10} insulin. After stimulation with either HMR 1964 or human insulin, an approximately twofold increase in Shc phosphorylation was observed (Fig. 3). Exactly the same results were obtained regarding the phosphorylation level of the 66-kDa Shc isoform (Fig. 3). It is worth noting that the 52-kDa Shc isoform exhibited the most prominent phosphorylation, whereas the association with the IGF-I receptor was observed nearly exclusively for the 66-kDa isoform. This result may have reflected the differential kinetics of Shc/IGF-I receptor interaction and phosphorylation and/or differential dephosphorylation of the two isoforms.

It has been reported that IGF-I receptor internalization regulates signaling via the MAPK but not the IRS-1 pathway (24). Taking into account a high rate of internalization of the analogs Asp^{B10} insulin and HMR 1153 and the stronger interaction with the Shc proteins, we anticipated that these two analogs might induce a strong activation of the p42/44 MAPK in the K6 myoblasts. Activation of the MAPKs was assessed by monitoring the phosphorylation state of these proteins using phosphospecific MAPK antiserum that detects tyrosine-phosphorylated ERK-1 and -2. The data shown in Fig. 4 indicate that K6 cells expressed both the 44- and the 42-kDa MAPK isoforms. The phosphorylation of both isoforms was strongly activated after

TABLE 2
In vivo analysis of cell proliferation by immunohistochemistry: Ki-67 labeling indices in mammary glands

		HMR 1964		HR 1799 (human insulin; 2 × 20 IU/kg)
	Controls	2 × 20 IU/kg	2 × 50 IU/kg	
n	29	23	14	19
Metestrus	5.06 ± 2.38	5.13 ± 1.69	5.10 ± 3.59	5.48 ± 3.46
Diestrus	6.11 ± 2.39	4.74 ± 2.23	5.87 ± 2.00	6.47 ± 4.04
Estrus	6.03 ± 0.73	6.06 ± 2.90	5.94 ± 3.17	5.99 ± 2.83

Data are means ± SD of the indicated number of animals. In vivo proliferation on mammary glands was determined as described in RESEARCH DESIGN AND METHODS. The labeling index indicative for proliferation was expressed as the percentage of positive labeled nuclei per total number of counted nuclei (at least 1,000).

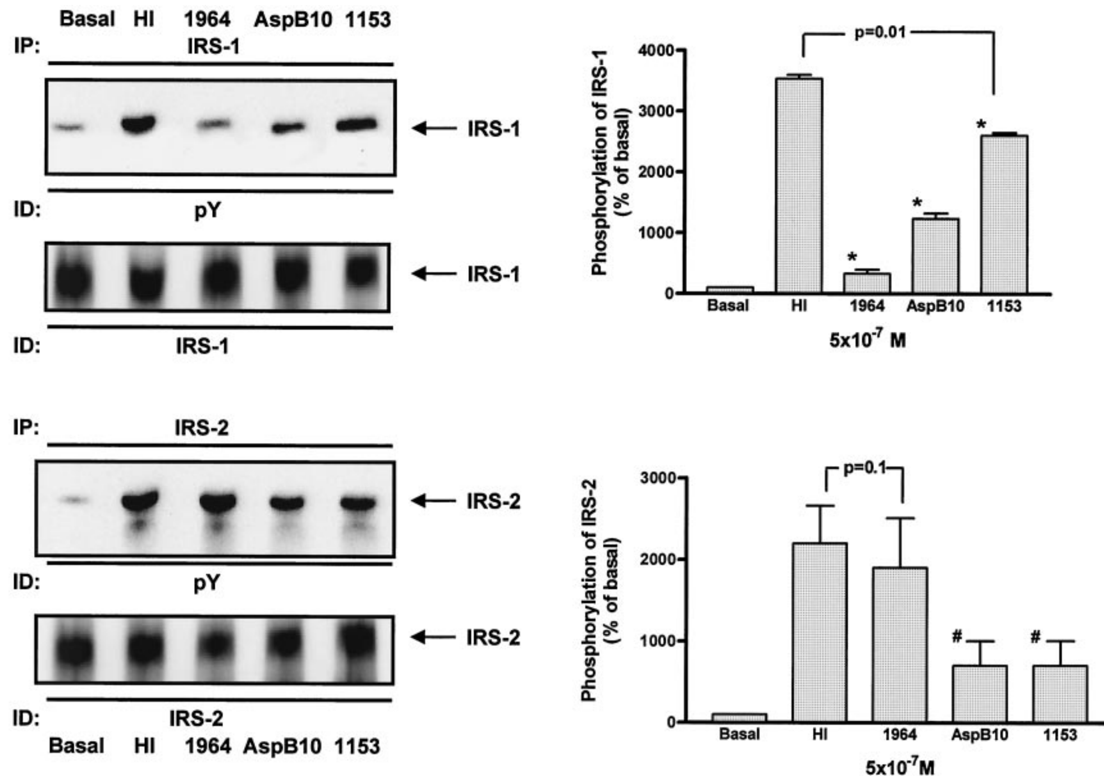

FIG. 6. Tyrosine phosphorylation of IRS proteins in response to insulin and insulin analogs in K6 myoblasts. Cells were stimulated as outlined in Fig. 1 and both IRS-1 and IRS-2 were immunoprecipitated (IP) and processed for immunoblotting with anti-phosphotyrosine antibodies. Filters were stripped and reprobed with anti–IRS-1 or anti–IRS-2 antiserum, respectively, to ensure equal loading. Signals were quantified using LumiImager software. Data are means ± SE of 3–4 separate experiments. ID, immunodetection; pY, phosphotyrosine. *P < 0.05 vs. basal and all other stimulated values; #P < 0.05 vs. human insulin (HI) and 1964.

cells were stimulated with HMR 1153 or Asp^{B10} insulin. Quantification of the data showed a 7.5- and 6-fold activation for ERK-1 and -2, respectively, after treatment with HMR 1153. Asp^{B10} insulin was less potent than HMR 1153 but still produced a significantly higher response compared with human insulin and HMR 1964. The most important finding was that the latter analog produced the lowest response of MAPK activation, which was significantly different from human insulin.

Effect of insulin and insulin analogs on cellular proliferation in vitro and in vivo. The ERK-1/2 signaling pathway plays a critical role in the regulation of cellular proliferation and differentiation (25). We therefore determined cellular proliferation of K6 myoblasts in response to human insulin and the different analogs by monitoring DNA synthesis using the incorporation of BrdU and a highly sensitive chemiluminescence immunoassay. Serum-starved myoblasts responded with a fourfold increase in BrdU incorporation when stimulated with 10% FCS or IGF-I for 16 h (Fig. 5). Both Asp^{B10} insulin and HMR 1153 were equipotent, inducing a two- to threefold increase in DNA synthesis, but were significantly less potent than IGF-I. The smallest response (~50%) was observed for both human insulin and HMR 1964 (Fig. 5). These two molecules were significantly less potent than Asp^{B10} insulin and HMR 1153. Thus, the growth-promoting activity of the insulin analog HMR 1964 was identical to that of human insulin and was completely consistent with the insulin-like activation of the Shc/MAPK cascade by this analog, again similar to insulin.

In addition, an investigation on the mitogenic effect of regular insulin and the analog HMR 1964 was performed in vivo on mammary glands of female rats treated with different dosages of the hormones during a 12-month toxicity study using Ki-67 immunohistochemistry. As shown in Table 2, no significant difference was observed among groups treated with high dosages of HMR 1964 (2 × 20 IU/kg and 2 × 50 IU/kg) or human regular insulin (2 × 20 IU/kg) in comparison with control groups (HMR 1964 placebo solution). This result was in complete agreement with the in vitro analysis of the proliferative potential of these insulins.

Tyrosine phosphorylation of IRS-1/2 in rat and human myoblasts. The data obtained so far in the study suggested that the analogs HMR 1153 and Asp^{B10} insulin strongly activated the MAPK pathway via a prominent stimulation of Shc phosphorylation. In contrast, human insulin and the analog HMR 1964 exerted a much weaker effect on this pathway. To further dissect the signaling properties of the different insulin analogs, we determined their effects on the tyrosine phosphorylation of IRS-1/2 in K6 myoblasts. The cells were stimulated with insulin or insulin analogs for 10 min as described above, and IRS-1 or IRS-2 was immunoprecipitated and immunoblotted with anti-phosphotyrosine antibodies. As shown in Fig. 6, human insulin produced the strongest tyrosine phosphorylation of both IRS-1 and IRS-2, whereas the analogs Asp^{B10} insulin and HMR 1153 induced a less-pronounced stimulation of both IRS proteins. It is interesting that the analog HMR 1964 produced only a marginal phosphorylation of

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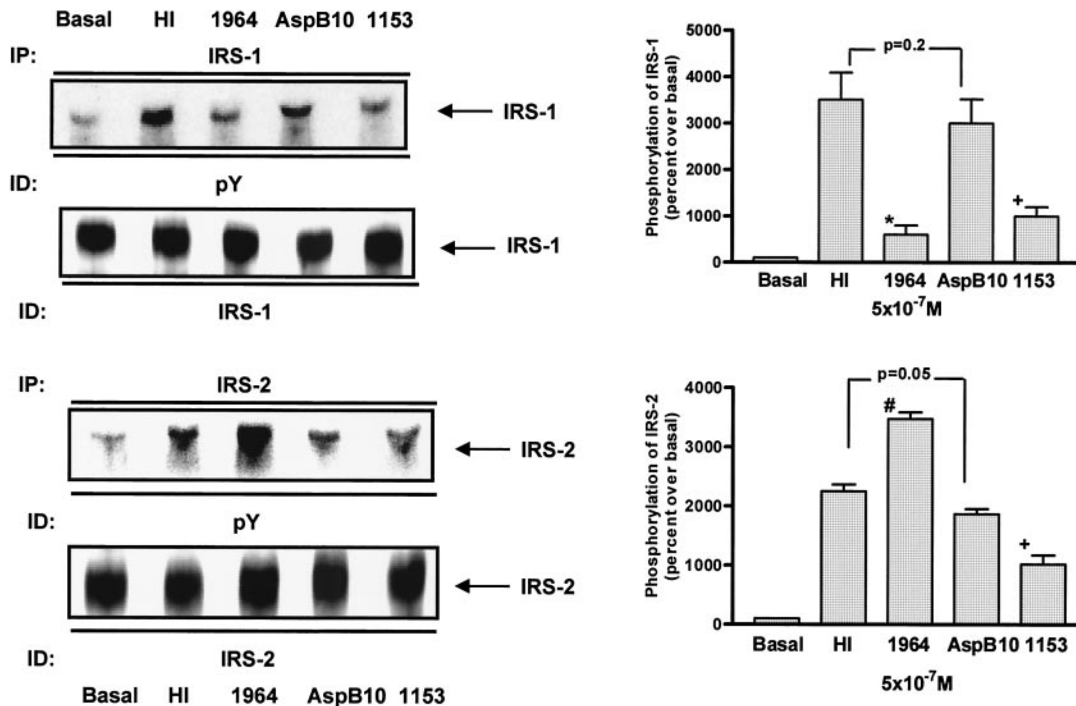

FIG. 7. Tyrosine phosphorylation of IRS proteins in proliferating human skeletal muscle cells in response to insulin and insulin analogs. Human myoblasts (10^6 cells/dish) were cultured as described in RESEARCH DESIGN AND METHODS and subjected to serum starvation for 4 days. The cells were then stimulated with the different insulins as described in Fig. 1. IRS-1 and IRS-2 were immunoprecipitated (IP) and processed for immunoblotting with anti-phosphotyrosine antibodies. Stripping, reprobing with anti–IRS-1 and anti–IRS-2 antibodies, and quantification of the signals was performed as described in Fig. 6. Data are means ± SE of 4–5 separate experiments. ID, immunodetection; pY, phosphotyrosine. *$P < 0.05$ vs. basal and all other stimulated values; #$P < 0.05$ vs. human insulin (HI); +$P < 0.05$ vs. all other values.

IRS-1 but produced a prominent phosphorylation of IRS-2, similar to that seen after stimulation with human insulin (Fig. 6). Quantification of the Western blots (Fig. 6, *right*) demonstrated a nearly 30- and 20-fold response after treatment with human insulin for IRS-1 and IRS-2, respectively. The analog HMR 1964 exerted a marginal twofold effect on the activation of IRS-1, but induced a 20-fold increase of IRS-2 phosphorylation, which made it as effective as human insulin (Fig. 6).

Because the predominant stimulation of IRS-2 by a modified insulin molecule was unexpected, we repeated these experiments in a different cell system. Proliferating primary human skeletal muscle cells were stimulated with human insulin and the different analogs using exactly the same protocol as outlined above for the K6 myoblasts. The tyrosine phosphorylation of IRS-1 and IRS-2 was analyzed by immunoblotting (Fig. 7). As seen before, human insulin produced a prominent phosphorylation of both IRS-1 and IRS-2, with the analog Asp^{B10} insulin being equipotent to the regular insulin molecule. However, the insulin analog HMR 1964 produced a marginal phosphorylation of IRS-1 and a strong tyrosine phosphorylation of IRS-2 that was even significantly higher than that seen after stimulation of cells with human insulin (Fig. 7). We therefore concluded that the preferential activation of IRS-2 by the insulin analog HMR 1964 represents a unique property of this molecule that is also observed in human cells.

Tyrosine phosphorylation of IRS-1/2 in adult cardiomyocytes. It might be argued that the predominant activation of IRS-2 by HMR 1964 is mediated by the IGF-I receptor and thus could be limited to myoblastic cells. We therefore studied the tyrosine phosphorylation of IRS-1 and IRS-2 in response to the different insulin analogs in primary adult cardiomyocytes. These cells express a higher level of insulin receptors but a much lower level of IGF-I receptors (by ~2:1) than myoblasts and have been extensively used in our laboratory for studies on insulin signaling and insulin action (19,26,27). All experiments were conducted under the same conditions as described before for K6 cells and human myoblasts. As shown in Fig. 8, in this cell system human insulin also produced a strong phosphorylation of both IRS-1 and IRS-2, whereas Asp^{B10} insulin and HMR 1153 were less effective. Again, tyrosine phosphorylation of IRS-1 was only marginally activated by HMR 1964 (twofold). However, this analog produced an 18-fold increase of the tyrosine phosphorylation of IRS-2, reaching the same level as that seen with human insulin (Fig. 8). These data confirmed that the novel analog HMR 1964 preferentially signals along the IRS-2 pathway, in both myoblasts and differentiated muscle cells.

Differential activation of IRS-1 and IRS-2 by the insulin analogs may affect downstream signaling intermediates such as Akt and GSK-3, two serine/threonine kinases involved in propagating the insulin signal to the glucose transporter and glycogen synthesis machinery (28,29). As presented in Fig. 9, a prominent activation of both Akt and GSK-3 was observed with essentially no difference between insulin and all analogs tested.

Despite a normal activation of downstream kinases, the decreased IRS-1 activation by HMR 1964 might limit the metabolic activity of this analog. To address this issue, we measured the stimulation of 3-*O*-methylglucose transport by this analog in direct comparison with human insulin, Asp^{B10} insulin, and HMR 1153 using the adult cardiomyo-

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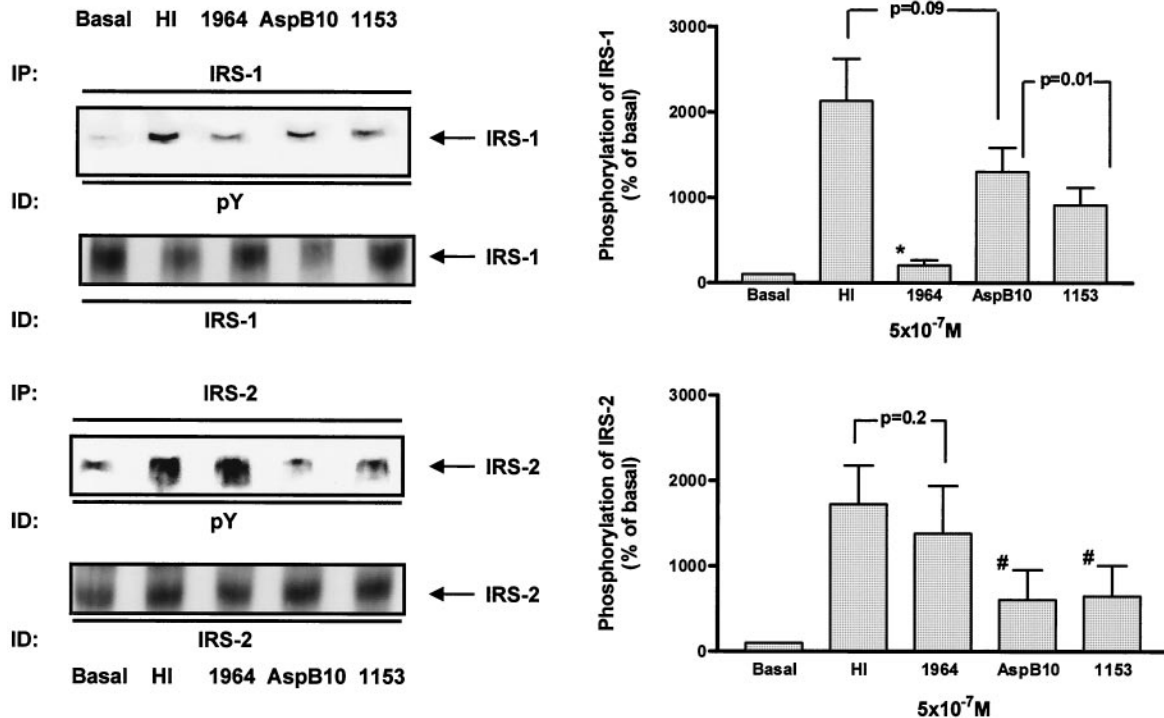

FIG. 8. Tyrosine phosphorylation of IRS proteins in adult rat cardiomyocytes in response to insulin and insulin analogs. Freshly isolated cardiomyocytes (4×10^5 cells) were stimulated for 10 min with 500 nmol/l of insulin and insulin analogs. Cells were then lysed and processed for immunoprecipitation (IP) and immunoblotting of IRS-1/2, as described in Fig. 6. Quantification was performed using LumiImager software. Data are means ± SE of 4 separate experiments. pY, phosphotyrosine. *$P < 0.05$ vs. basal and all other stimulated values; #$P < 0.05$ vs. human insulin (HI) and 1964.

cyte system. As presented in Fig. 10, the initial rate of glucose transport was increased 3- and 4.4-fold in response to 5 and 500 nmol/l insulin, respectively. Essentially the same response was observed after treatment of cells with HMR 1964 (Fig. 10). Thus, regular activation of IRS-2 and limited activation of IRS-1 appears to be suffi-

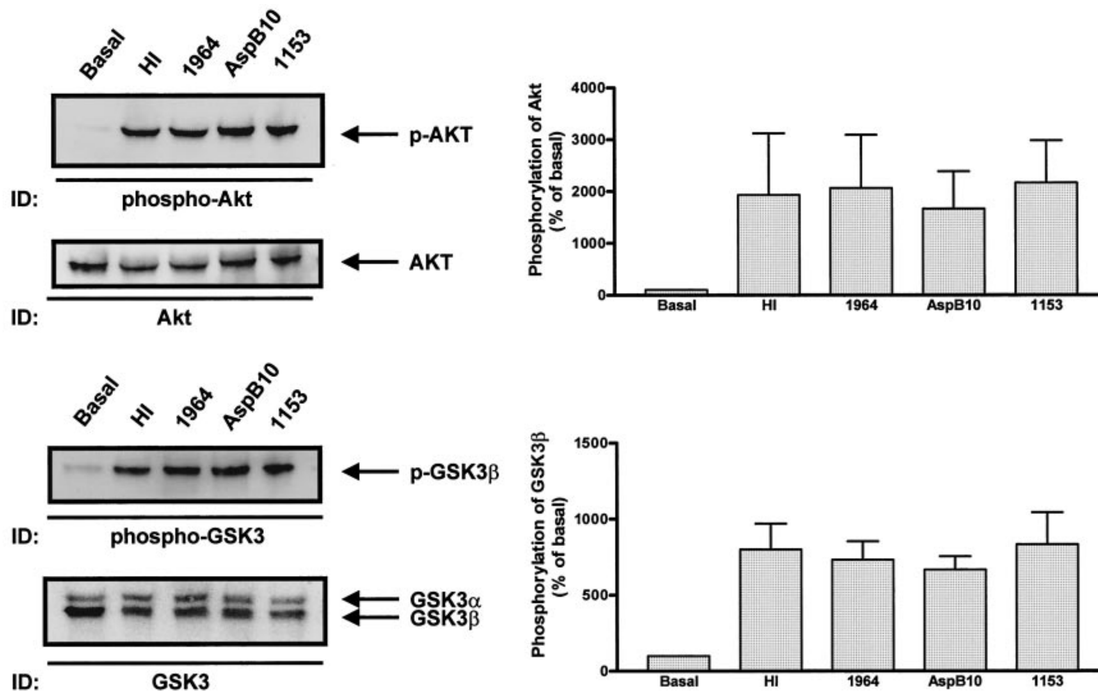

FIG. 9. Serine phosphorylation of Akt and GSK-3 in adult rat cardiomyocytes in response to insulin and insulin analogs. Cells were stimulated with the different insulins as described in Fig. 8 and lysed. Cellular proteins were separated by SDS-PAGE and immunoblotted with phospho-Akt or phospho-GSK3 antibodies, stripped, and reprobed with Akt or GSK-3 antibodies using ECL detection. Signals were quantified using the LumiImager software. Data are means ± SE obtained from 3 separate experiments. HI, human insulin; ID, immunodetection. All stimulated values were significantly different from basal (at least $P < 0.01$) but not from each other.

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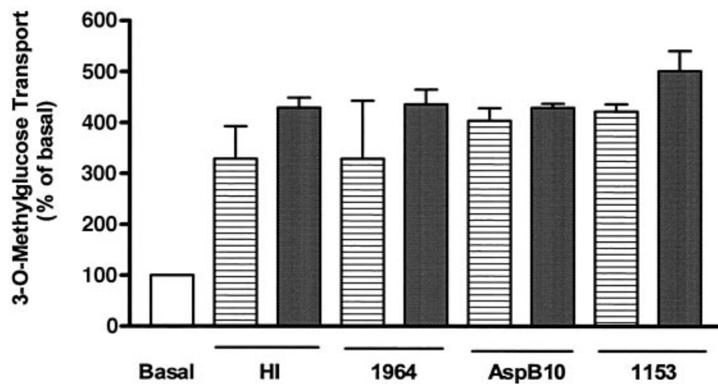

FIG. 10. Transport of 3-*O*-methylglucose in adult cardiomyocytes in response to insulin, HMR 1964, Asp^{B10} insulin, and HMR 1153. For this study, 4 × 10^5 cells/ml were incubated for 10 min in the absence (Basal) or presence of the indicated concentrations of insulin or insulin analogs. Initial rates of 3-*O*-methylglucose were then determined over a 10-s assay period as outlined in RESEARCH DESIGN AND METHODS. Data are means ± SE of 3–4 separate experiments. ▤, 5 nmol/l; ■, 500 nmol/l; HI, human insulin. All stimulated values were significantly different from basal ($P < 0.01$) but not from each other.

cient for propagating downstream signaling to glucose transporters in muscle cells. In agreement with the results obtained on the stimulation of Akt (Fig. 9), both Asp^{B10} insulin and HMR 1153 were equipotent to insulin in activating glucose transport (Fig. 10).

DISCUSSION

A key finding of the present investigation was the observation that the novel rapid-acting analog LysB3,GluB29 insulin (HMR 1964) was able to generate predominant activation of the IRS-2 signaling pathway in muscle cells, concomitantly exhibiting the same mitogenic and metabolic properties as regular human insulin. To our knowledge, this is the first report on the predominant activation of IRS-2 by an insulin analog. Modification of the B26−B30 region of the insulin molecule has been extensively used to produce insulin analogs with reduced self-association that are suitable as rapid-acting insulin molecules (3–5). However, modifications within this domain of the insulin molecule are known to increase the affinity of a given analog for the IGF-I receptor, consequently leading to enhanced mitogenic activity and a potential safety risk of these compounds when in long-term use (9,12,15,30). In the present study, we reassessed this concept by performing a detailed analysis of the signaling and mitogenic properties of the two analogs LysB3,GluB29 insulin (HMR 1964) and LysB3,IleB28 insulin (HMR 1153) in rat and human myoblasts expressing a high level of IGF-I receptors. Our data suggest that in addition to the binding affinity and occupancy time at the receptor (14), initial steps of receptor activation and/or processing may also contribute to triggering specific downstream signaling pathways by the insulin molecule.

Both IRS-1 and Shc have been implicated in the activation of the MAPK pathway by IGF-I and insulin (31,32), a signaling event with central importance for the control of cellular growth and differentiation by these hormones (33). More recently, a differential interaction of the phosphotyrosine-binding domains of Shc and IRS-1 with the IGF-I receptor has been reported (34), and a sustained tyrosine phosphorylation of Shc in response to IGF-I has been found to correlate with enhanced MAPK activation and growth of human neuroblastoma cells (35). As shown by Chow et al. (24), IGF-I receptor internalization is required for signaling via the Shc/MAPK pathway but not the IRS-1 pathway. Our data consistently showed a good correlation between the internalization of the insulin analogs and the activation of the MAPK pathway in the K6 myoblasts. Thus, HMR 1153 exhibited a three- to fourfold higher internalization when compared with HMR 1964, which in turn resulted in a three- to fourfold higher Shc phosphorylation and ERK-1/2 activation in response to HMR 1153. Furthermore, the low internalization rate of HMR 1964 correlated with a marginal activation of ERK-1/2 by this analog that was even lower than that induced by insulin. It has also been demonstrated that sustained receptor binding decreases endosomal insulin degradation, resulting in enhanced signaling from this intracellular compartment (36). This would explain the strong activation of MAPK by Asp^{B10} insulin, as this analog exhibits a moderate internalization combined with a very low degradation (Table 1). Recent reports have shown that activation of MAPK also correlates to internalization of epidermal growth factor receptors (37) and G protein−coupled receptors (38). Preliminary data in our laboratory suggest a potential difference between insulin and IGF-I receptors. Thus, inhibition of internalization in adult cardiomyocytes only marginally affected MAPK activation by regular insulin, with a much stronger reduction in the case of Asp^{B10} insulin and HMR 1153, two ligands with higher affinity for the IGF-I receptor.

Human insulin and HMR 1964 produced a moderate activation of the Shc/MAPK pathway and DNA synthesis in the K6 cells. It is important to note that under the same conditions, insulin induced a prominent activation of both IRS-1 and IRS-2. Thus, our data support the notion that tyrosine phosphorylation of Shc, most likely leading to formation of the Shc-Grb2 complex, represents the key step in IGF-I receptor signaling to the MAPK pathway (32,35). It is also evident that the prominent activation of IRS-2 by HMR 1964 did not mediate MAPK activation. This observation is consistent with the view that IRS-2 is of major importance for mediating metabolic events (39–41). It is interesting that the autophosphorylation of the IGF-I receptor in response to HMR 1964 was comparable with that seen after stimulation of cells with Asp^{B10} insulin; however, the association of Shc with the IGF-I receptor as well as downstream activation of MAPK and DNA synthesis were much stronger after stimulation with Asp^{B10} insulin, with the analog HMR 1964 exhibiting effects comparable to those with human insulin. Consistent with this finding, we demonstrated in a 12-month toxicity study in rats treated with high dosages of HMR 1964 that the in vivo growth-promoting activity of this analog was identical to that of regular insulin; in vivo data concerning the proliferative activity of HMR 1153 are presently lacking. In

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agreement with our findings, Sobke et al. (42) observed essentially the same effect of HMR 1964 and human insulin on DNA synthesis using the mammary epithelial cell line MCF10. Furthermore, HMR 1153 produced a very strong autophosphorylation of the IGF-I receptor but the same association with Shc when compared with Asp^{B10} insulin. These observations support our hypothesis that the insulin/receptor interaction may control the specificity of downstream signaling by a combination of several mechanisms. In addition to binding, this may include the internalization and processing of the ligand receptor complex, the half-life of the receptor complex, and a differential phosphorylation pattern of the IGF-I/insulin receptor in response to the different analogs. Certainly, the latter possibility is highly speculative, but it fits with the observation that Shc and IRS-1 use functionally distinct mechanisms to recognize tyrosine phosphorylated receptors (34,43). Additional work is now required to dissect the molecular mechanism leading to the differential signaling by insulin analogs at the level of the IGF-I/insulin receptor. In the present study, we used high concentrations of insulin and insulin analogs to ensure occupancy of IGF-I receptors. At the present stage, we do not know if the differential effects of insulin analogs are also operative at low physiological insulin concentrations, and this limitation of our study must be taken into account.

Human insulin exerted the most prominent activation of IRS-1 and IRS-2 in both rat and human myoblasts and adult cardiomyocytes. HMR 1153 and Asp^{B10} insulin were much less effective at this level of the insulin-signaling cascade, despite a twofold higher proliferative activity of these two analogs in the K6 myoblasts. Surprisingly, the analog HMR 1964 produced only a marginal activation of IRS-1 in the three cell systems but a prominent activation of IRS-2 that was even significantly different from that of regular insulin in the human myoblasts. It might be argued that this is a specific property of myoblastic cells expressing a high level of the IGF-I receptor. However, we demonstrated here that HMR 1964 produced mainly an activation of IRS-2 in adult rat cardiomyocytes, a cell expressing a high level of insulin receptors compared with myoblasts (26). Recent evidence suggests a tissue-specific role of the IRS proteins in insulin signaling, with IRS-1 playing a prominent role in skeletal muscle and IRS-2 playing such a role in liver (44). Consistent with this, it has been shown that IRS-1 deficiency in mice can be well compensated for by increased IRS-2 phosphorylation in liver, but not in muscle, where the responses to insulin-induced phosphatidylinositol 3-kinase activation, glucose transport, and protein synthesis were significantly impaired in comparison with wild-type mice (45). Thus, it seems reasonable to assume that regular activation of IRS-2 combined with a minor phosphorylation of IRS-1 is sufficient to produce a full metabolic response in the adult cardiomyocyte, as HMR 1964 was equipotent to insulin in activating glucose transport in these cells. Consistent with this, despite differential activation of IRS-1 and IRS-2 by the different analogs, all analogs produced full activation of Akt and GSK-3, suggesting the possibility that a certain level of IRS-1 and/or IRS-2 phosphorylation is sufficient for downstream signaling to Akt/GSK-3. This result differs from our findings on the activation of the Shc/MAPK cascade, where we found a tight correlation among Shc phosphorylation, activation of MAPK, and DNA synthesis. The molecular basis of the predominant activation of IRS-2 by HMR 1964 is presently unknown. However, current evidence points to the view that IRS-1 and IRS-2 can interact with tyrosine phosphorylated receptors via multiple independent binding motifs (46) and functionally distinct mechanisms (47), probably contributing to signal diversification. These data support our hypothesis that signaling specificity through different IRS proteins may be accomplished during interaction with the activated receptor by distinct receptor phosphorylation patterns in response to the different insulin analogs.

In summary, our data suggest that the mitogenic and metabolic properties of insulin analogs may result from a complex series of initial receptor/ligand interactions involving binding affinity, the timing-dependent specificity, receptor internalization, and, potentially, a specific pattern of receptor phosphorylation. The unique property of HMR 1964, showing a predominant activation of IRS-2 combined with a marginal activation of the MAPK mitogenic pathway, clearly indicates that the primary structure of the insulin molecule contains sufficient information to control hormonal action at the downstream level. Selective activation of IRS-2 by an insulin analog may be of central interest for future strategies to optimize insulin therapy.

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