The Paired Basic Amino Acid-cleaving Enzyme 4 (PACE4) Is Involved in the Maturation of Insulin Receptor Isoform B

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To cite this version:

Imène Kara, Marjorie Poggi, Bernadette Bonardo, Roland Govers, Jean-François Landrier, et al.. The Paired Basic Amino Acid-cleaving Enzyme 4 (PACE4) Is Involved in the Maturation of Insulin Receptor Isoform B: an opportunity to reduce the specific insulin receptor-dependent effects of insulin-like growth factor 2 (IGF2). Journal of Biological Chemistry, 2015, 290 (5), pp.2812 - 2821. 10.1074/jbc.M114.592543 . inserm-01478251

HAL Id: inserm-01478251
https://inserm.hal.science/inserm-01478251
Submitted on 27 May 2020

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The Paired Basic Amino Acid-cleaving Enzyme 4 (PACE4) Is Involved in the Maturation of Insulin Receptor Isoform B

AN OPPORTUNITY TO REDUCE THE SPECIFIC INSULIN RECEPTOR-DEPENDENT EFFECTS OF INSULIN-LIKE GROWTH FACTOR 2 (IGF2)*

Received for publication, July 3, 2014, and in revised form, December 10, 2014 Published, JBC Papers in Press, December 19, 2014, DOI 10.1074/jbc.M114.592543

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** This work was supported by INSERM, INRA, and Aix-Marseille Université.
1 Recipient of a grant from the Ministère de la Recherche et de l’Enseignement Supérieur.
2 Supported by Prostate Cancer Canada Grants D2013-8, 2012-951, and TAG2014-02 and Canadian Cancer Society Research Institute Grant 701590.
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5 The abbreviations used are: IR, insulin receptor; dec-RVKR-cmk, proprotein convertase inhibitory decanoyl-Arg-Val-Lys-Arg-chloromethylketone; IGF, insulin-like growth factor; ML, multi-Leu peptide inhibitor (Ac-LLLLRVKR-NH2); PEG8-ML, PEGylated multi-Leu peptide inhibitor; PC, proprotein convertase; Phac-RVR-4-Abma, proprotein convertase inhibitor phenylacetyl-Arg-Val-Arg-4-amidobenzylamide; PKB, protein kinase B; proIR, proform of the insulin receptor.

Gaining the full activity of the insulin receptor (IR) requires the proteolytic cleavage of its proform by intra-Golgi furin-like activity. In mammalian cells, IR is expressed as two isoforms (IRB and IRA) that are responsible for insulin action. However, only IRB transmits the growth-promoting and mitogenic effects of insulin-like growth factor 2. Here we demonstrate that the two IR isoforms are similarly cleaved by furin, but when furin activity is reduced, IRB is matured by PACE4.

Results:
IRA and IRB are similarly matured by furin, but when furin activity is reduced, IRB is matured by PACE4.

Conclusion:
Proprotein convertase inhibition can selectively reduce IRA maturation and its signaling.

Significance:
This can be considered as a new opportunity to target IRA signaling in cancer.

Background:
The insulin receptor exists as two isoforms: IRA and IRB.

The mature insulin receptor (IR) is a heterotetrameric transmembrane protein composed of two disulfide-linked dimers, each of which has an amino-terminal ligand-binding α subunit and a carboxyl-terminal tyrosine kinase active β subunit (for review, see Ref. 1). The α and β subunits are synthesized as a single precursor polypeptide (proIR) that is cleaved after being transported to the Golgi compartments (2) on the carboxyl side of the tetrabasic amino acid sequence (Arg-Lys-Arg-Arg) located at the junction between the two subunits (3, 4). The physiological importance of proteolytic proIR maturation was demonstrated in patients who were suffering major insulin-resistant diabetes because of an Arg735→Ser substitution at the proIR processing site (5, 6). Moreover, a processing defect in the proIR was observed in the livers of a particular animal model for diabetes (7).

In this study, we show that IRA is cleaved by furin to yields a mature form (mIRA) containing the α subunit and a carboxyl-terminal fragment (ΔCIRB) containing the β subunit. IRA and IRB are similarly cleaved by furin, but when furin activity is reduced, IRB is matured by PACE4.
α subunit that is close to the aforementioned proreceptor processing site (three residues before the carboxyl terminus of the α-chain). The relative abundance of the two splice variants is regulated in a tissue-specific manner (for review, see Ref. 16). IRA is ubiquitously expressed and is preponderant in fetal and cancer tissues, whereas IRB is predominantly expressed in target tissues of the insulin metabolic effects. The two IR isoforms present subtle differences in terms of activation and signaling. IRA is a high affinity receptor for insulin but also for IGF2 (17, 18) and IGF1 (19). It displays faster internalization and recycling kinetics (20, 21), which could explain its higher propensity for signal proliferation and survival in comparison with IRB (16). Furthermore, selective insulin signaling through IRA and IRB that regulates transcription of insulin and glucokinase genes was demonstrated in pancreatic β cells (22). This selectivity was believed to result from IR isoform-mediated signaling from different plasma membrane microdomains (23, 24).

A seminal study has demonstrated that both proIR isoforms are similarly matured in vitro by recombinant furin (25). However, an exhaustive statistical analysis of furin cleavage sites interpreted within the three-dimensional crystal structure of the furin catalytic domain revealed that the actual furin cleavage site stretches far beyond the tetra-basic consensus sequence and contains up to 20 amino acids (26). This sequence encompasses 10 amino acids of IRB exon 11, indicating that the IRA and IRB furin recognition sites are different.

We have investigated the cellular proteolytic maturation of IRA and IRB. Our data clearly show that even though furin is predominantly responsible for the maturation of the two insulin proreceptor isoforms, it is not essential for IRB maturation. IRB maturation can be achieved at the cell surface by PACE4. We took advantage of this difference in IRA and IRB maturation to demonstrate that it is possible to reduce the mitogenic signals emanating from the IR by inhibiting IRA maturation.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The PC inhibitors decanoyl-Arg-Val-Lys-Arg-chloromethylketone (dec-RVKR-cmk) and tripeptide derivative phenylacetyl-Arg-Val-Arg-4-amidinobenzylamide (Phac-RVR-4-Amba) were purchased from Merck. The PC inhibitor multileucine peptide (ML) and its cell-impermeable PEGylated derivative (PEG8-ML) were previously described (27). Insulin receptor antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) (β chain, sc711; α chain, sc7953; phosphorylated β chain (Tyr1162/1163), sc25103). PKB and Erk1/2, pan and phosphospecific antibodies were purchased from Cell Signaling Technology (Danvers, MA). The His$_{6}$ antibody was purchased from GeneTex (Irvine, CA). Flag M2 antibody, insulin, and IGF2 were purchased from Sigma-Aldrich. Phospho-Erk1/2 and phospho-PKB ELISA kits were purchased from eBioscience (San Diego, CA). Anti-furin monoclonal (Mon-152) and anti-P7C7 polyclonal antibodies were produced by John Creemers.

**Expression Vectors**—The expression vectors for IRA, IRB, an uncleavable form of IR, furin, PC7, PACE4, PC5/6A, PC5/6B, α1-PDX, and EK4 were all described elsewhere (22, 28–32). The vectors for human furin and PC7 shRNA were described previously (33).

**Cell Culture and Transfection**—The human cell lines HeLa, LoVo, HEK293, and the murine cell line 3T3L1 were maintained in culture as described by ATCC. The adipocyte differentiation of 3T3L1 cells was performed as previously described (34). Transient cell transfection was performed with Polyjet reagent (SignaGene Laboratories, Rockville, MD) as specified by the manufacturer.

**Enzymatic Deglycosylation**—N-Linked carbohydrate residues were removed by incubating the cell lysates for 2 h at 37 °C with 1000 units of either peptide-N-glycosidase F or endoglycosidase H (New England Biolabs, Beverly, MA) as described by the manufacturer.

**Cell Surface Protein Isolation**—The cell surface protein isolation kit was used as described by the manufacturer (Pierce). In brief, LoVo cells were washed four times with ice-cold PBS. The membrane-impermeable biotinylation reagent EZ-Link Sulfo-NHS-SS-Biotin was added to a final concentration of 100 μM, and the cells were incubated on ice for 30 min. The biotinylation reaction was stopped by adding the quenching solution followed by two PBS washes. The cells were lysed, and labeled proteins were isolated with NeutrAvidin-agarose resin. Proteins were eluted from the resin and analyzed by immunoblot.

**Immunoblot**—Total cell lysates were separated on 4–12% gradient NuPAGE gels with MOPS SDS running buffer (Invitrogen) and transferred to polyvinylidene fluoride membranes. The membranes were blocked for 1 h in 5% BSA solution, and the proteins were immunodetected with the appropriate antibodies. Image acquisition was performed by using a chemiluminescent CCD imager Image Quant LAS 4000 (GE Healthcare). A densitometric analysis of the bands corresponding to the proform and mature form of IR (proIR and IR, respectively) was performed with the ImageQuant TL software (GE Healthcare), and IR proteolytic maturation was calculated as the IR/proIR ratio.

**Measuring Furin Activity**—Intra-Golgi furin activity was monitored with the GRAPfurin reporter construct (35). 48 h after transient transfection, the cell medium was replaced by uncolored DMEM for an incubation period of 5 h. The medium was centrifuged at 20,000 x g, mixed with the alkaline phosphatase substrate para-nitrophenyl phosphate in a 96-well plate, and incubated 1 h for color development, and then the absorbance was read at 405 nm. The values were normalized to those obtained from empty vector-transfected cells.

**Glut4 Translocation Assay**—The fluorescence-based assay for the detection of cell surface GLUT4 levels has been previously described (36).

**2-Deoxy-d-glucose Uptake Assay**—Glucose uptake activity of 3T3L1 adipocytes was measured by the chemiluminescent assay (37) using Glucofax kit as described by the manufacturer (Yelen, Ensuies la Redonne, France).

**Real Time PCR Analysis**—Total RNA was extracted using a Nucleospin RNA kit (Macherey-Nagel, Hoerdt, France), cDNA was synthesized from 0.5 μg of RNA using. Moloney murine leukemia virus reverse transcriptase (Invitrogen) was used for PCR amplification. RT-PCRs were performed on the LightCycler 480 instrument (Roche Applied Science) using the Eva Green MasterMix (Euromedex, Souffelweyersheim, France). The comparative Ct method (2^{-ΔΔCT}) was used to calculate
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RESULTS

In the Absence of Furin, IRB Proteolytic Maturation Is Higher Than That of IRA—The use of a computational tool for predicting furin cleavage sites (PiTou software) that was developed based on the functional characterization of the 20-residue recognition sequence motif (38) revealed that both proIR isoforms can be cleaved by furin at the unique expected site (Arg735 for IRB and Arg723 for IRA) with similar efficiencies. Consistent with this prediction, comparable amounts of immature and mature IRA and IRB forms were detected in the lysates of HeLa cells that independently overexpressed either of these isoforms (Fig. 1A); consequently, the proteolytic maturation of both IR isoforms, which was calculated as the IR/proIR ratio, was not significantly different. However, when IRA and IRB were overexpressed in furin-deficient LoVo cells, the mature IRB was detected in larger amounts than that of IRA, and their proform quantities were comparable (Fig. 1B). As a consequence, the IRB proteolytic maturation was significantly greater (2.17 ± 0.75-fold) than that of IRA. It should be noted that the overexpressed IRA had still matured a bit. The re-expression of furin in LoVo cells increased the maturation (increases in the mature form and decreases in proIR) of both proIR isoforms to the same level (Fig. 1C), rendering the maturation of IRA and IRB comparable. Consistent with this result, a furin knockdown in HeLa cells more substantially reduced the proteolytic maturation of IRA than that of IRB (Fig. 1D), so the maturation of IRB was significantly greater (1.61 ± 0.27-fold; \( p = 0.015; \ n = 7 \)) than that of IRA. It should be emphasized that the transfection efficiency was ~50–60% (data not shown) and that furin detection was made on the whole cell preparation, which explains the apparent low reduction of the furin amount by the shRNA. As a logical consequence of the significant reduction of the mature IRA isoform in furin knockdown cells, the amount of its phosphorylated form in response to insulin treatment was proportionally reduced (Fig. 1E). These data demonstrate that furin is responsible for most of the proIR proteolytic maturation. However, in the absence of furin, IRA and IRB maturation is differently regulated.

In the Absence of Furin, IRB Proform Is Exposed at the Cell Surface—Immunoblot analysis revealed that the proIR expressed in LoVo cells appeared as a doublet (Fig. 1B), and furin overexpression led to the disappearance of the upper band of the doublet (Fig. 1C). This doublet appeared also upon furin

the relative differences in mRNA expression. The acidic ribosomal phosphoprotein P0 (Rplp0) was used as housekeeping gene. Primers sequences are available upon request.

Cell Proliferation Assay—Cell proliferation was determined using the DELFIA proliferation assay kit (PerkinElmer Life Sciences) according to the manufacturer’s instructions. Briefly, HEK293 cells were transfected in 6-well plate, 12 h after they were detached using Accutase (GE Healthcare Europe, Velizy-Villacoublay, France) and seeded into a 96-well white walled plate at a density of 4000 cells/well. After complete adhesion, cells were serum-starved (0.5% FBS) overnight and then treated with Phac-RVR-4-Amb or vehicle for 4 h before the addition of BrdU and insulin or IGF2. Cells were incubated for an additional 16 h, fixed, and incubated with anti-BrdU labeled with europium for 90 min. Time-resolved fluorescence was measured at 340-nm excitation and 615-nm emission.

Statistical Analyses—All data were analyzed with GraphPad Prism software. The applied test was described in the figure legends or in the text. Statistical significance was set at \( p < 0.05 \).
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The cellular localization of the IR proform. A, HeLa cells were transfected with an empty vector or with vectors coding for IRB or for a mutated form of IRB (IRBnc) that cannot be cleaved by PC. B, LoVo cells were cotransfected with IRB together with an empty vector or with a furin expression vector (+), and the cells were surface-biotinylated. The total (left panel) and cell surface (right panel) proteins were treated for immunoblot. C, lysates of LoVo cells expressing IRB alone or in association with furin were mock treated or treated for deglycosylation with peptide-N-glycosidase F (pNGase) or endoglycosidase H (endoH). D, mRNA levels of IR, IRA, and IRB were measured by quantitative PCR in 3T3L1 adipocytes (data are expressed as means ± S.D.; n = 5; the mean basal IR expression was set at 100). E, 3T3L1 adipocytes were treated or not with dec-RVKR-cmk (RVKR; 5 μM) for 12 h. The IR was analyzed by immunoblot with IRβ antibody.

knockdown in furin-expressing cells (Fig. 1D). Furthermore, the proform of a mutated IR that cannot be proteolyzed by PCs was also detected as a doublet (Fig. 2A), indicating that this doublet originates from inefficient proIR proteolytic maturation. In LoVo cells, the slow migrating proform of IR (the upper band of the proIR doublet) was exposed at the cell surface, as demonstrated by cell surface biotinylation (Fig. 2B). As expected, furin overexpression reduced the amount of cell surface-exposed proIR and increased the amount of mature IR. Deglycosylation experiments confirmed that the slow migrating proform of IR crossed the Golgi apparatus because it was endoglycosidase H-resistant, whereas the endoglycosidase H-sensitive fast migrating proIR was in the endoplasmic reticulum (Fig. 2C). As a control, both proIR forms were deglycosylated by peptide-N-glycosidase F treatment. The 3T3L1 adipocytes express comparable amounts of IRA and IRB mRNA (Fig. 2D) and produce detectable amount of endogenous IR. Treating these cells with the general potent irreversible PC inhibitor (dec-RVKR-cmk) reduced IR maturation and enabled the slow migrating proform of IR to appear (Fig. 2F), indicating that cell surface exposure of the IR proform in response to PC inhibition is not caused by a side effect of IR overexpression. These results indicate that when proIR is not efficiently cleaved by furin in the Golgi apparatus, it is still routed to the cell surface. It is important to note that furin knockdown in HeLa cells reduced more efficiently the proteolytic maturation of IRA than that of IRB (Fig. 1D) and also increased the amount of the slow migrating proform of IRA to a greater extent than that of IRB. This observation is in accordance with the more exclusive involvement of furin in the proteolytic maturation of IRA than that of IRB. We also note that in LoVo cells, although IRA and IRB are differently matured, there was no obvious difference in the amount of their slow migrating proforms (Fig. 1, B and C), suggesting that in these cells the furin-independent maturation of IR is rather low.

In the Absence of Furin, IRB Undergoes Convertase-dependent Proteolytic Maturation—We investigated whether a PC other than furin could be responsible for IRB maturation. Treating furin-deficient LoVo cells with the general PC inhibitor dec-RVKR-cmk reduced the proteolytic maturation of IRB, whereas the low level of IRA maturation was barely reduced (Fig. 3A).

This result demonstrates that a PC other than furin is responsible for IRB proteolytic maturation. However, IR maturation that persists after dec-RVKR-cmk treatment suggests that, in our conditions, either dec-RVKR-cmk does not inhibit all the PCs responsible for the proteolytic maturation of IR or that the remaining maturation of IR is PC-independent. To clarify these issues, we used the PC suicide substrate inhibitors α1-PDX (29) and EK4 (30) (two engineered α1-antitrypsin serpins with different mutation in the recognition site). In HeLa cells, EK4 proved to be a slightly better furin inhibitor than α1-PDX (Fig. 3B). Interestingly, the maturation of IRA, which is more exclusively under the control of furin than that of IRB, was similarly reduced by α1-PDX and EK4 (Fig. 3C), whereas the maturation of IRB was significantly more reduced by EK4 than by α1-PDX. We conclude that α1-PDX and EK4 are both effective inhibitors of the furin-dependent maturation of IR, but in addition, EK4 inhibits other PCs involved in IRB maturation. In LoVo cells, overexpression of EK4 reduced the furin-independent maturation of IRA and IRB (Fig. 3D) meaning that, in the absence of furin, a PC activity is still responsible for at least a part of the low level of IRA maturation.

These results further emphasize that the proteolytic maturation of IRA is more exclusively under the control of furin than that of IRB. They also show that in the absence of furin, the residual maturation of IRB (and to a lesser extent that of IRA) involves another PC activity.

PACE4 Is Responsible for the Cell Surface Furin-independent Maturation of IRB—ProIR proteolytic maturation was analyzed in LoVo cells overexpressing the PCs that exhibit a substantial degree of functional redundancy with furin (39). Only the overexpression of PACE4 more efficiently increased the maturation of the IRB than that of IRA (Fig. 4, A and B). In addition, a reduction in the amount of the slow migrating proform of IRB was observed (Fig. 4B). This result supports the involvement of PACE4 in the enhanced maturation of the IRB proform in the absence of furin. Unexpectedly, overexpressed PC7, PC5/6A, and PC5/6B increased the maturation of both proIR isoforms as
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FIGURE 3. The involvement of PCs other than furin in IR maturation. A, LoVo cells expressing either IRA or IRB were treated or not with dec-RVKR-cmk (RVKR; 5 μM) for 12 h. The IR was analyzed by immunoblot with IRβ antibody, and the lower panel is a long exposure of the immunoblot showing the mature form of IR. B, furin activity was measured in the media of HeLa cells overexpressing GRAPfurin together with empty vector, α1-PDX (PDX), or EK4 expression vectors (data are expressed as means ± S.D.; n = 5; the mean basal furin activity was set at 100. *, p < 0.05; **, p < 0.001; analysis of variance, Bonferroni test). C, HeLa cells were cotransfected with either IRA or IRB together with an empty plasmid, α1-PDX (PDX), or EK4. Left panel, the IR was analyzed by immunoblot with IRβ antibody. Right panel, inhibitory effect of α1-PDX (PDX) or EK4 on the maturation of IRA and IRB in HeLa cells (data are means ± S.D. expressed as the percentage of the basal maturation; n = 6; *, p < 0.05; **, p < 0.001; analysis of variance; Bonferroni test). D, LoVo cells were cotransfected with either IRA or IRB together with an empty plasmid or EK4, and IR was analyzed as described for A.

efficiently as furin (Fig. 4A). However, these PCs cleaved both proIR isoforms with the same efficiency, which does not support their role in the furin-independent maturation of the IRB proform. Furthermore, in LoVo cells, knockdown of PC7, which is the PC that is most similar to furin in terms of primary structure and subcellular localization, did not change the maturation of the IRB proform (Fig. 4C). PC5/6 is not expressed in HeLa cells (Table 1) and thus cannot be responsible for the differential maturation of IRA and IRB observed after furin knockdown (Fig. 1D). Taken together, these data suggest that the overexpression of PC7 and PC5/6 allows a proteolytic maturation of IR that is insignificant in physiological conditions.

PACE4 is constitutively secreted into the extracellular matrix (39) and localizes at the cell surface and in the extracellular matrix (40). We investigated whether the PACE4-dependent maturation of IR occurs in the secretory pathway or at the cell surface.

Incubation of LoVo cells with the conditioned medium from HEK293 cells overexpressing the PC inhibitor EK4 reduced IRB maturation but not substantially that of IRA (Fig. 4D). Furthermore, the multi-Leu peptide PACE4 inhibitor and its cell-impermeable derivative, two potent and relatively selective inhibitors (20-fold specificity over furin) (27), were equally efficient in reducing the furin-independent maturation of IRB (Fig. 4E), whereas the processing of IRA was marginally reduced. However, multi-Leu peptide PACE4 inhibitor did not reduce IR processing in LoVo cells overexpressing furin (Fig. 4F). These results show that the PC-dependent and furin-independent maturation of IRB occurs at the cell surface. Furthermore, the proteolytic maturation of IRB was increased by PACE4 added extracellularly (Fig. 4G), and in that case the slow migrating proform of IRB disappeared completely, whereas that of IRA was only slightly reduced. As a control, PC5/6A added extracellularly similarly increased the maturation of both IR isoforms (Fig. 4H). These results demonstrate that PACE4 possesses the ability to selectively process the cell surface-exposed proform of IRB into its mature form. Taken together, our results argue that the furin-independent maturation of IRB involves PACE4 and takes place at the cell surface.

Furin Inhibition Reduces IRA Maturation—Most cells simultaneously express both IR isoforms. We investigated whether the proportion of mature IRA and IRB could be modified by manipulating the PC activity. Differently tagged IR isoforms were used to distinguish IRA from IRB in cotransfection experiments. LoVo cells were cotransfected with IRAGFP and IRB in the absence or presence of furin. As expected, only mature IRB was detected in the absence of furin, and furin expression increased the maturation of both IRAGFP and IRB (Fig. 5A, left panel). A similar experiment was performed by using HeLa cells cotransfected with IRAGFP and IRB in the absence or presence of the furin-specific shRNA. Furin knockdown reduced the amount of mature IRAGFP but not that of IRB (Fig. 5A, right panel). These experiments indicate that reducing the furin activity preferentially diminished the proteolytic maturation of IRA over that of IRB.

To avoid cotransfecting IR with furin-specific shRNA, we tested commercial PC inhibitors for their ability to alter the maturation of both IR isoforms to different extents. The dose effect of the cell-permeable PC inhibitor Phac-RVR-4-Amba (41) on the proteolytic maturation of both proIR isoforms was investigated in HeLa cells that independently overexpressed IRA and IRB. The maximal inhibition of IRA maturation was obtained with a concentration of Phac-RVR-4-Amba as low as 0.5 μM, whereas a concentration 10 times higher was necessary to inhibit IRB maturation to the same extent (Fig. 5B). As a result, with 0.5 μM of Phac-RVR-4-Amba, the maturation of IRA was significantly more reduced than that of IRB (reduction by 53 ± 5 and 22 ± 7% of the maturation of IRA and IRB...
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FIGURE 4. The importance of PACE4 in IR maturation. A, LoVo cells were cotransfected with IRA or IRB expression vector together with an empty vector (−) or with the indicated PC expression vector. B, LoVo cells were cotransfected with IRA or IRB together with an empty vector or with a PACE4 expression vector. C, LoVo cells were cotransfected with IRB and PC7-specific shRNA (shPC7). D, LoVo cells expressing IRA or IRB were incubated for 12 h with conditioned media from HEK293 cells transfected with an empty plasmid or with EK4 expression vector. E, same as D except that cells were incubated with 50 μM of multi-Leu peptide PACE4 inhibitor (ML) or its cell-impermeable derivative (PEG8-ML). F, LoVo cells expressing furin and IRA or IRB were treated with 50 μM of ML. G, same as D except that cells were incubated for 8 h with conditioned media from HEK293 cells transfected with a PACE4 expression vector. H, same as E except that cells were incubated with conditioned media from HEK293 cells transfected PCs/6A expression vector. Each blot is representative of three independent experiments. The IR was analyzed by immunoblot with IRα (A) or IRβ antibody, and PC7 position is indicated by PC7. The middle panel in C and the lower panels in D and E are long exposures of the immunoblots showing the mature form of IR (IRβ).

TABLE 1
mRNA levels of PCs in different cell lines

|         | Rplp0 | Furin | PACE4 | PC5/6 | PC7 |
|---------|-------|-------|-------|-------|-----|
| Hek293  | 17.0 ± 0.3 | 32.1 ± 0.3 | 21.1 ± 0.2 | 23.4 ± 0.2 | 23.1 ± 0.3 |
| HeLa    | 15.2 ± 0.3 | 32.5 ± 0.2 | 23.6 ± 0.4 | Not detected | 23.8 ± 0.4 |
| LoVo    | 14.8 ± 0.4 | Not tested | 21.2 ± 0.4 | 24.3 ± 0.5 | 23.0 ± 0.4 |

respectively; n = 6; p < 0.05; analysis of variance, Bonferroni test).

When the two IR isoforms were coexpressed in HeLa cells, Phac-RVR-4-Amba treatment reduced the maturation of IRA and allowed the detection of its slow migrating proform, whereas the maturation of IRB was poorly altered (Fig. 5C). These results show that treatment with Phac-RVR-4-Amba is as effective as furin-specific knockdown at reducing proteolytic maturation of IRA without unduly modifying that of IRB.

Inhibition of IRA Maturation Differentially Alters IR-dependent Insulin and IGF2 Signaling—We investigated the impact of the PC inhibitor Phac-RVR-4-Amba on the signaling triggered by IR agonists (insulin and IGF2). In HEK293 cells, IGF2 more efficiently stimulated the phosphorylation of IRA than that of IRB, whereas the phosphorylation of both IR isoforms was similarly stimulated by insulin (Fig. 6A). These results are compatible with a higher IGF2 affinity for IRA in comparison with IRB. As expected, in cells overexpressing both IR isoforms, insulin and IGF2 increased IR phosphorylation, and Phac-RVR-4-Amba slightly reduced the maturation of IR (Fig. 6, B and D). However, Phac-RVR-4-Amba prevented more efficiently the phosphorylation of IR induced by IGF2 than that induced by insulin, which is in accordance with its greater inhibitory effect on the maturation of IRA than on that of IRB. In HEK293 cells, phosphorylation of endogenous Erk1/2 resembled that of IR, i.e. it was stimulated by insulin, and IGF2 and Phac-RVR-4-Amba prevented more efficiently the phosphorylation of Erk1/2 induced by IGF2 than that induced by insulin (Fig. 6, B and C). In HeLa cells, IR overexpression allowed insulin to stimulate the phosphorylation of endogenous PKB, which was not reduced by Phac-RVR-4-Amba treatment (Fig. 6, D and E). Interestingly, PKB phosphorylation was stimulated by IGF2 even in the absence of IR overexpression, but Phac-RVR-4-Amba treatment somehow still reduced this phosphorylation. Taken together, these data demonstrate that Phac-RVR-4-Amba, which is more efficient in reducing the maturation of IRA than that of IRB, reduces some of the signals that are triggered by IGF2 without altering insulin signaling.

Treatment with Phac-RVR-4-Amba Reduces Cellular Effects of IGF-2—Because treatment with Phac-RVR-4-Amba reduced intermediate signaling in IR mitogenic pathways, we analyzed whether this compound could actually inhibit cell proliferation. The proliferative effect of IGF2 on HEK293 cells did not neces-
sitate the overexpression of IR, although it was significantly more pronounced in IR-overexpressing cells (Table 2). Contrarily, the proliferative effect of insulin was only observed in HEK293 cells that overexpressed IR. In all situations, the proliferation induced by IR agonists was abolished by Phac-RVR-4-Amba treatment (Table 2). The impact of Phac-RVR-4-Amba treatment on the metabolic response to IGF2 and insulin was studied in 3T3L1 adipocytes taken as a model of insulin target cells. As expected for cells that express both IR isoforms (Fig. 2D), Phac-RVR-4-Amba treatment reduced the maturation of IR and the IGF2- and insulin-dependent phosphorylation of PKB (Fig. 7A) in a manner essentially comparable with those described previously (Fig. 6, B, D, and E). In addition, the IGF2-dependent GLUT4 plasma membrane translocation and glucose uptake were significantly reduced by Phac-RVR-4-Amba treatment, whereas those stimulated by insulin were not altered (Fig. 7, B and C). These results demonstrate that Phac-RVR-4-Amba treatment reduces the proliferative and metabolic effects of IGF2, whereas it only reduces the proliferative effect of insulin.

DISCUSSION

The present study demonstrates that the IRB proform exposed at the cell surface is efficiently processed to its mature form by PACE4. Furthermore, these data highlight the possibility of reducing IRA maturation and its associated mitogenic signals without unduly affecting the metabolic signals emanating from IRB.

Our data do not detract from the previously reported importance of furin in the maturation of IR (9–11). Indeed, we used knockdown and re-expression experiments to demonstrate that furin is the primary PC involved in the maturation of IRA and IRB. However, the specific furin deficiency (LoVo cells or specific knockdown) or PC inhibition (chemical inhibitors or overexpression of PDX and EK4) led to a more important reduction in the maturation of IRA than that of IRB. These results emphasize that the proteolytic maturation of IRA is more under the control of furin than that of IRB.

As reported by others (28), we confirm that when proIR is not efficiently cleaved by furin in the Golgi apparatus, it is still routed to the cell surface. However, in addition to this, by using PC inhibitors that do not penetrate the cell (treatment with EK4 or with the cell impermeable multi-Leu peptide PACE4 inhibitor), we provide arguments indicating that the proform of IRB...
exposed at the cell surface is prone to a PC-dependent (and furin-independent) maturation. We also show that PACE4 (overexpressed or added extracellularly) is the only PC that has the peculiar property of increasing the maturation of IRB without changing that of IRA. These results are in accordance with the location and action of PACE4 at the cell surface and in the extracellular matrix (40, 42). In brief, when the intracellular furin-dependent maturation of IR is reduced, both proIR isoforms are exposed at the cell surface where only the proform of IRB is efficiently matured by PACE4. Along the same lines, angiopoietin-like 3 was shown to be matured intracellularly by furin action and at the cell surface by PACE4 (43). The ability of PACE4 to process the IRB proform at the cell surface could explain the efficient maturation of IR in furin knock-out mouse livers (13). Indeed, in the liver, PACE4 is highly expressed (13, 44), and IRB is the preponderant IR isoform (16).

Speculation can be made on the elements that are potentially involved in the differential cleavage of the two IR isoforms by PACE4. The absence or presence of exon 11 in IRA and IRB, respectively, can directly alter the recognition of IR by PACE4. Indeed, as determined for furin (26), PACE4 recognition motifs could stretch far from the cleavage site, and in the case of IR, the recognition motif could encompass the exon 11. Our result showing that the two proIR isoforms exposed at the cell surface are similarly cleaved by extracellularly of PC5/6A suggests that their accessibility to the extracellular PCs is not fundamentally different. However, the two IR isoforms are believed to be localized in different plasma membrane microdomains (23, 24); therefore, their membrane microenvironment can be different. Because PACE4 localizes at the cell surface through interaction with heparin sulfate proteoglycans (40) or tissue inhibitor of metalloproteinases (42), the composition of the IR membrane microenvironment could affect the accumulation of PACE4 and thereby regulate IR cleavage. The fact that PACE4 cleaves IRB at the cell surface and not in the constitutive secretory pathway could be related to the preference of PACE4 for cleaving substrates at neutral pH (14, 40). It is also conceivable that PACE4 responsible for IR maturation is inactive in the secretory pathway. Indeed, it was previously suggested that PACE4 activation could take place at the cell surface (45).

In cells expressing the two IR isoforms, a specific furin knockdown efficiently reduces the maturation of IRA and poorly alters that of IRB, which results in a lower quantity of mature IRA. To simplify our experimental system, we tested commercial PC inhibitors for their ability to reduce the proteolytic maturation of IRA and not that of IRB, and we found that treatment of cells with low doses of Phac-RVR-4-Amba allows reaching that goal. The ability of this PC inhibitor to differently alter the maturation of the two IR isoforms was exploited to modulate IR-dependent metabolic and mitogenic signaling. The phosphorylation of IRA and IRB is similarly stimulated by insulin, whereas IGF2 more effectively stimulates the phosphorylation of IRA than that of IRB. This finding is consistent with previous data demonstrating that IGF2 has a higher affinity for IRA than IRB (17, 18) and implies that IRA is responsible for the mitogenic signals emanating from IR (16). In cells expressing both IR isoforms, the IGF2-stimulated phosphorylation of IR, was reduced by Phac-RVR-4-Amba treatment, whereas the insulin effect was poorly altered. Furthermore, Phac-RVR-4-Amba treatment significantly reduces the IR-dependent phosphorylation of Erk1/2 triggered by IGF2, whereas the phosphorylation of Erk1/2 and PKB triggered by insulin was not compromised. These results validate the idea that it is possible to orient IR signaling through a pharmacological manipulation of proIR isoforms maturation; by reducing IRA maturation without modifying that of IRB, it is possible to reduce the IR mitogenic signals (triggered by IGF2 and emanating from IRA).
without modifying the metabolic signals (triggered by insulin). Clearly, the unprocessed IRA that is exposed at the cell surface does not interfere with IR signaling, which is in accordance with data reporting that the signaling ability of the proIR exposed at the cell surface is severely reduced (28, 46–48). Treatment with Phac-RVR-4-Amba not only reduces intermediate signaling in IR mitogenic pathways but also inhibits IR-dependent cell proliferation, which is in line with previous data demonstrating that furin inhibition attenuates critical properties of tumor cells (35, 49–51). The measure of GLUT4 plasma membrane translocation and glucose uptake using 3T3 L1 adipocytes taken as a model of insulin target cells confirm that the metabolic effects of insulin are not altered by Phac-RVR-4-Amba treatment.

As suggested, exacerbated IRA signaling may represent an important mechanism of resistance to various anticancer therapies and should therefore be considered to be a target in cancer therapy (16). Our results indicating that furin inhibition is an effective strategy to block signaling through IRA without altering the metabolic effects of insulin will pave the way for new opportunities to target the IRA signaling in cancer in a specific fashion.

Acknowledgments—We are indebted to M. R. Knittler (Friedrich Loeffler Institute, Greifswald, Germany) for sharing human furin- and PC7 shRNA-expressing vectors and R. Leduc (Université de Sherbrooke, Québec, Canada) and E. Clauser (INSEERM L970, Paris, France) for sharing their EK4 and furin uncleavable IR expressing vectors, respectively. We thank N. Vidal (Yelen, Enses La Redonne, France) for measuring glucose uptake.

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The Paired Basic Amino Acid-cleaving Enzyme 4 (PACE4) Is Involved in the Maturation of Insulin Receptor Isoform B: An Opportunity to Reduce the Specific Insulin Receptor-Dependent Effects of Insulin-Like Growth Factor 2 (IGF2)

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J. Biol. Chem. 2015, 290:2812-2821. doi: 10.1074/jbc.M114.592543 originally published online December 19, 2014

Access the most updated version of this article at doi: 10.1074/jbc.M114.592543

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