Accurate and Efficient Cleavage of the Human Insulin Proreceptor by the Human Proprotein-processing Protease Furin

CHARACTERIZATION AND KINETIC PARAMETERS USING THE PURIFIED, SECRETED SOLUBLE PROTEASE EXPRESSED BY A RECOMBINANT BACULOVIRUS*

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Maturation of the insulin proreceptor in a late Golgi compartment requires cleavage at an Arg-Lys-Arg-Arg processing site, suggesting involvement of furin, a transmembrane serine protease of the Kex2 family of processing enzymes. A genetically engineered secreted, soluble form of human furin (ss-furin), expressed by infection of insect cells with a recombinant baculovirus, was purified to near homogeneity. ss-Furin exhibited rapid and efficient cleavage of both isoforms of the human insulin proreceptor in solubilized extracts of cultured mammalian cells expressing proreceptor cDNA. Proreceptor cleavage occurred at the physiological processing site as judged by the effects of mutations in this site on cleavage by purified ss-furin. Moreover, purified ss-furin exhibited specificity for proreceptor cleavage identical to that of the endogenous insulin proreceptor-processing enzyme. Furin thus displays the properties expected of an insulin proreceptor-processing enzyme in that it (i) cleaves the proreceptor efficiently and at the correct site; (ii) exhibits the same specificity in processing variant proreceptors as the endogenous enzyme; (iii) appears to be localized in the correct secretory compartment; and (iv) has the same broad pattern of tissue distribution as the insulin proreceptor.

Mature human insulin receptor (hIR) is an α-β-β-α disulfide-linked tetramer composed of extracellular α-subunits that confer insulin binding and membrane-spanning β-subunits that transduce a signal to the cell interior by activation of the β-subunit tyrosine kinase (1, 2). The α- and β-subunits are synthesized as a single proreceptor (IPR) polypeptide that is cleaved, after transport to a late Golgi compartment (3), carboxylic to the tetrabasic sequence (Arg-Lys-Arg-Arg) which lies at the junction between amino-terminal α-subunit and carboxy-terminal β-subunit sequences (4, 5). Similar processing sites are found in structurally related proreceptors including the insulin-like growth factor-I receptor, the insulin receptor-related receptor, and the hepatocyte growth factor receptor (6–8). Processing of human IPR (hIPR) is extremely efficient in vivo. The cellular processing enzyme(s) is not saturated even with 100-fold overproduction of hIPR (>10⁶ receptors/cell), which is achieved routinely by transfection (9).

Two isoforms of the insulin receptor differ by the presence or absence of a 12-residue segment 3 residues amino-terminal to the proreceptor processing site, the result of alternative splicing of exon 11 in the hIPR pre-mRNA (hIR−11 lacks and hIR+11 has exon 11) (10). The two are expressed in a tissue-specific fashion. hIR−11 alone appears to be expressed in lymphocytes, whereas both hIR−11 and hIR+11 are expressed in liver, kidney, and muscle.

Physiological importance for proteolytic maturation of hIPR was established with the discovery of two sisters presenting extreme insulin-resistant diabetes, apparently due to substitution of Ser for Arg⁷²⁶ at the P⁷ position of the proreceptor processing site (11, 12). Additionally, in an animal model for diabetes, a defect in proreceptor processing was indicated from an increase in the insulin proreceptor to receptor ratio in ketotic rats (13). It is unclear precisely how processing affects receptor function, and the hIR−11 and hIR+11 isoforms may differ in this regard (14). Proreceptors acquire insulin binding capacity before proteolytic cleavage in the late Golgi, indicating that processing is not absolutely required for binding (15). Epstein-Barr virus-transformed lymphocytes derived from one patient with the Ser substitution at the P⁷ position in the processing site exhibited only proreceptors, presumably the -11 isoform, at the cell surface. These cells exhibited dramatically reduced insulin binding and signal transduction. In contrast, when a series of substitutions was made in the hIPR+11 type receptor (see below), uncleaved proreceptors exhibited near normal insulin affinity, although somewhat higher levels of insulin were required for activation of receptor tyrosine kinase activity. Thus, the absence of processing may have a more drastic effect on the function of the -11 isoform than on that of the +11 isoform.

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The abbreviations used are: hIR, human insulin receptor; IPR, insulin proreceptor; hIPR, human insulin proreceptor; HIV-I, human immunodeficiency virus type I; ss-furin, secreted, soluble furin; Endo H, endoglucoasaminidase H; Boc-RVRR-MCA, tert-butyloxycarbonyl-Arg-Val-Arg-MCA; AMC, 7-amino-4-methylcoumarin; bp, base pairs; MES, 2-[N-morpholino]ethanesulfonate; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-HCl-buffered saline; anti-IRp antibodies, rabbit anti-hIR β-subunit polyclonal antibodies; CHO, Chinese hamster ovary.

2 Residues amino-terminal to a cleaved bond are designated P₁, P₂, P₃, and P₄, etc.

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Mutations created in the hIPR processing site revealed that Arg was essential for cleavage at the P₁ and P₄ positions. Substitution of Ala for Lys at P₃ or Arg at P₃ had little or no effect on cleavage of hIPR (16). This pattern of specificity (~Arg-Xaa-Xaa-Arg~) matches that of the processed protease, furin (17-19). Human furin was the first member of a family of mammalian processing enzymes identified by homology to yeast Exo2 protease, a Ca²⁺-dependent serine protease that cleaves the α-mating pheromone precursor at Lys-Arg sites (20-22). Expression of the fur gene, which encodes furin, appears to be ubiquitous, although the level of expression is developmentally modulated (23). Colocalization with TGN38 and failure to redistribute to the endoplasmic reticulum in the presence of brefeldin A suggest localization of furin in the trans-Golgi network (24), although it should be cautioned that experiments performed only with high levels of furin expression. The nearly ubiquitous expression of both furin and hIPR, the correlation between furin localization and the cellular location of hIPR cleavage, and the nature of the hIPR processing site make furin a likely candidate for the physiological hIPR-processing enzyme. Furin has been implicated in processing the proforms of several lipid enveloped viruses including fowl plague hemagglutinin (25) and the 160-kDa glycoprotein from HIV-I (26) and so is a potential target for antiviral drugs. Knowledge of furin’s physiological roles will become important in assessing the consequences of chronic or acute inhibition of its enzyme.

This paper presents biochemical evidence that furin processes the insulin proreceptor. Using recombinant baculovirus expression, we have expressed a secreted, soluble form of human furin (ss-furin) and purified the enzyme. We have found that the purified enzyme cleaves both isoforms of insulin proreceptor efficiently and in a Ca²⁺-dependent manner. Finally, site-directed mutagenesis of the hIPR processing site has demonstrated that purified ss-furin exhibits the same specificity as the physiological hIPR-processing enzyme.

**EXPERIMENTAL PROCEDURES**

*Materials—Fast-flow Q-Sepharose was from Pharmacia Biotech Inc.; N-glycanase from Genzyme and endogluccosaminidase H (Endo H) from Boehringer Mannheim; S9 and H5 insect cells, and wild type baculoviral DNA were from Invitrogen Corporation. Free EXCELL 401 medium was from Jackson Laboratories. Grace’s and Ham’s F-12 media were from Life Technologies, Inc. (tetr-Butoxyanilinyl-Arg-Val-Arg-Gly-methylcoumarin amide (Boc-RVRR-MCA) and 7-amino-4-methylcoumarin (AMC) were from Peptides Int.

Cloning Human fur cDNA—Oligonucleotide primers corresponding to the 5’ end of exon S and the 3’ end of exon T of the human fur gene (8) were used to amplify a 300-bp fragment from a human KB cell cDNA library in the plasmid vector pCD (27) (supplied by Takashi Yokota, Jackson Laboratories). When cells reached 70% confluence (60 x 10⁶ cells/flask), medium was removed, and monolayers were infected with bac-fur595 stock virus at a multiplicity of infection of two. After 1 h, the inoculum was replaced with fresh EXCELL 401 medium. After an additional 90 h, medium was harvested by centrifugation at 1,000 x g for 15 min and dialyzed against four changes of 6 liters of buffer A (50 mM Tris-HCl, pH 8.0, 1 mM o-phenanthroline, in 0.1 M KCl and 0.1 M CaCl₂). Purification of ss-Furin—In 50 ml the standard assay contained 20 nm NaMES, pH 7.0, 1 mM CaCl₂, 0.01% (w/v) Triton X-100, 0.5% (v/v) dimethyl sulfoxide, and either 100 μg Boc-RVRR-MCA or 150 μg of detergent-solubilized cell extracts. Reactions with Boc-RVRR-MCA were initiated by adding enzyme, incubated at 37 °C for various times, and terminated with 0.125% (w/v) ice-cold 0.1 M CaCl₂. AMC release was determined fluorometrically (32). A unit was defined as release of 1 pmol of AMC/min from Boc-RVRR-MCA under standard assay conditions. Furin was stable under assay conditions for >1 h. Reactions with detergent-solubilized cell extracts were terminated by adding ice-cold trichloroacetic acid to 6% (w/v), and precipitates recovered by centrifugation at 12,000 x g for 15 min were washed twice with ice-cold acetone, air dried, and solubilized in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (10 mM Tris-Cl, pH 6.8, 5 mM β-mercaptoethanol, 1% SDS, and 10% glycerol) at 100 °C for 5 min before immunoblotting.

Site-directed mutagenesis on double-stranded pBSIR96 was accomplished using the Double-Take mutagenesis kit (Stratagene Inc.). Oligonucleotides (PAN facility, Stanford University) used for mutagenesis were (5’ to 3’): CCTGCGTTTCGCGCTTGGCCTGGG (P,Ala), GC-GCGTTTCCG (P,Ala). Mutant clones were identified by restriction Site-directed mutagenesis on double-stranded pBSIR96 was accomplished using the Double-Take mutagenesis kit (Stratagene Inc.). Oligonucleotides (PAN facility, Stanford University) used for mutagenesis were (5’ to 3’): CCTGCGTTTCGCGCTTGGCCTGGG (P,Ala), GC-GCGTTTCCG (P,Ala), and GCCAAGGGACCGCTTTCCGAG (Pfla), and GCCAAGGGACG-

Transient Transfection of COS-1 Cells—COS-1 cells (3 x 10⁴) were transfected in 5 ml DMEM with 11 mg/ml polyethyleneimine, with 5 μg of wild type or mutant pSRa-hIR+11 expression constructs.
tor pMT3DSV2 (16). 72 h after transfection, cells were washed three times in phosphate-buffered saline (50 mM sodium phosphate, pH 7.4, 150 mM NaCl) and detergent-solubilized in 150 µl of 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, and 1% (v/v) sodium deoxycholate supplemented with protease inhibitors (final concentrations: 5 mM EDTA, 0.1 mM o-phenanthroline, 1 µg/ml leupeptin, 1 µg/ml antipain, 1 mM tosylphenylalanylchloromethyl ketone, 1 mM tosyllysyl chloromethyl ketone). Solubilized cell extracts were clarified by centrifugation at 12,000 g for 15 min, and the protein concentration of the supernatant was determined by the Bio-Rad protein assay. Detergent-solubilized cell extracts (150 µg of protein in equivalent to ~10 6 cells) were boiled 3 min after the addition of 0.25 volume of 5× SDS-PAGE sample buffer in preparation for immunoblot analysis.  

**Immunological/Immunochemical Methods—**Affinity-purified rabbit anti-hIR β-subunit polyclonal antibodies (anti-IRP) were as described (33). To obtain anti-furin antiserum, a lacZ-furin fusion was constructed from PUN-fur-595 into vector pUR278 (34). The resulting plasmid encoded a fusion protein (β-galactosidase-N-furin) with 366 residues of the fur cDNA with a stop codon after codon 595. The ss-furin coding sequence thus inserted a 1.3-kilobase pair SacII-SalI fragment of the fur cDNA into the lacZ gene. A recombinant baculovirus (hereafter, bac:fur-595), in which the ss-furin cDNA was placed under the control of the viral polyhedrin promoter, was isolated and plaque purified. Stocks of wild type (bac:wt) and bac:fur-595 virus were used to infect Hi5 cells, an ovarian cell line derived from the cabbage looper moth T. ni. Cells were grown in serum-free medium, and samples of media collected at times after infection were analyzed by SDS-PAGE (Fig. 2A). A 57-kDa Coomassie Blue-stained polypeptide that accumulated only in the medium of cells infected with bac:fur-595 virus (Fig. 2A) reacted specifically with affinity-purified polyclonal anti-furin antibodies as described under “Experimental Procedures.” In panels A and B, w = bac:wt, and f = bac:fur-595. Panel C, bac:wt and bac:fur-595 medium samples (5 µl) plus 100 µM Boc-RVRR-MCA, 200 mM Tris-Cl, pH 7.5, and 0.01% Triton X-100 containing either 1 mM CaCl₂ (+calcium) or 10 mM EDTA (+EDTA) were incubated for 60 min at 37 °C in a total volume of 50 µl. Incubations with medium from bac:wt-infected cells produced no AMC in 60 min either +calcium or +EDTA (both types of reactions are indicated with open squares).  

**RESULTS**  

**High Level Production of ss-Furin Using Recombinant Baculovirus**—Full-length cDNA encoding human preprofurin was obtained from a KB cell library by a two-step cloning procedure (see Fig. 1 and “Experimental Procedures”). To produce ss-furin, the cDNA was modified by introducing a translational stop codon after codon 595. The ss-furin coding sequence thus retained all sequences conserved in the Kex2 family of processing enzymes, the subtilisin domain and the contiguous P-domain (21, 22, 30). A recombinant baculovirus (hereafter: bac:fur-595), in which the ss-furin cDNA was placed under the control of the viral polyhedrin promoter, was isolated and plaque purified. Stocks of wild type (bac:wt) and bac:fur-595 virus were used to infect Hi5 cells, an ovarian cell line derived from the cabbage looper moth T. ni. Cells were grown in serum-free medium, and samples of media collected at times after infection were analyzed by SDS-PAGE (Fig. 2). A 57-kDa Coomassie Blue-stained polypeptide that accumulated only in the medium of cells infected with bac:fur-595 virus (Fig. 2A) reacted specifically with affinity-purified polyclonal anti-furin antibodies (Fig. 2B), confirming the identity of this species as ss-furin. Appearance of this band coincided with the appearance of a calcium-dependent proteolytic activity that cleaved the fluorogenic peptide substrate Boc-RVRR-MCA (Fig. 2A). Activity was first detected at 40 h after infection and increased linearly until at least 89 h after infection.  

**Purification of ss-Furin from Insect Cells**—Due to both the high level of expression of ss-furin driven by the bac:fur-595 virus and the ability to passage Hi5 cells in serum-free medium, the specific activity of ss-furin in the medium of cells infected with the recombinant virus (Table I) was 20–30-fold higher than obtained by expression in cultured mammalian cells.
cells\(^3\) (17, 18). As a result, purification of ss-furin from the medium of Hi5 cells infected with bac:fur-595 virus required only a 5-fold enrichment. Dialysis of the medium was required to remove a low molecular weight contaminant that prevented binding of the enzyme to Q-Sepharose. Selective elution of medium of Hi5 cells infected with bac:fur-595 virus required 17.8 kDa (Fig. 3B) and reacted specifically with anti-furin antibodies (data not shown). The enzyme in fraction 4 was judged to be $\geq 92\%$ pure by Coomassie Blue staining. Amino acid analysis was consistent with the expected amino acid composition of ss-furin.

**Active-site Titration of Purified ss-Furin**—Although most serine proteases do not exhibit rate-limiting cleavage of the acylenzyme with amide substrates, Kex2 protease was found to do so with a peptidyl methylcoumarin amide, permitting active-site titration by measuring the pre-steady-state burst of AMC release (32). ss-Furin also exhibited a pre-steady-state burst of AMC release in rapidly quenched reactions containing a saturating concentration of Boc-RVRR-MCA (400 $\mu$M) (Fig. 4A). With 10.4 pmol of ss-furin, as determined by quantitative amino acid analysis (data not shown), extrapolation of the linear phase of hydrolysis (500 ms-60 s) revealed a 6.6-pmol burst, indicating that the enzyme in fraction 4 was $\sim 60\%$ active.

Like preparations of furin derived from expression in mammalian cell culture (17, 18), cleavage of Boc-RVRR-MCA by ss-furin was Cys\(^\text{H_2S}\)-dependent. ss-Furin exhibited a $K_m$ of 18 $\mu$M and a $k_{cat}$ of 0.6 s\(^{-1}\) for Boc-RVRR-MCA yielding a $k_{cat}/K_m$ of 3.3 $\times 10^4$ M\(^{-1}\) s\(^{-1}\) (Fig. 4B), 33-fold lower than observed with purified Kex2 protease on the same substrate and 330-fold lower than ss-Furin on its best peptidyl-MCA substrate, acetyl-Pro-Met-Tyr-Lys-Arg-MCA (32). No hydrolysis was observed upon prolonged incubation ($\sim 1$ h) with ss-furin of acetyl-Pro-Met-Tyr-Lys-Arg-MCA at substrate concentrations up to 360 $\mu$M (data not shown). Conservatively, release of 5 pmol of AMC could have been observed, indicating an upper limit of 30 x$^{-1}$ s$^{-1}$ for $k_{cat}/K_m$ for ss-furin on this substrate.

**Cleavage of the Human Insulin Proreceptor by ss-Furin**—Initial attempts to assess the ability of ss-furin to cleave hIPR were made using medium from cells infected with the bac:fur-595 virus as a source of enzyme. CHO-IR cells, Chinese hamster ovary cells that stably express the hIR+11 isoform at a level 100-fold higher than the endogenous receptor, were the source of hIPR. Clarified extracts were prepared from CHO-IR cells by detergent solubilization followed by centrifugation at 12,000 $\times$ g. Affinity-purified polyclonal antibodies (anti-IR\(\beta\) antibodies) that recognize both hIPR and the mature p-subunit (Fig. 2). This pattern was essentially unaltered by incubation at 37 $^\circ$C for 30 min with buffer alone or with medium from bac:wt-infected cells (Fig. 5, lanes 2 and 3). Incubation of the CHO-IR extracts with medium from bac:fur-595-infected cells (18 units of ss-furin/reaction) resulted in disappearance of the 190-kDa proreceptor and the concomitant appearance of an

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**TABLE I**

| Step        | Protein | Volume | Activity | Specific activity | Yield |
|-------------|---------|--------|----------|------------------|-------|
| I. Medium   | 10.88   | 375    | 353,600  | 32,500           |       |
| II. Dialysate| 6.90    | 375    | 252,000  | 36,500           |       |
| III. Q-Sepharose | 1.40 | 360    | 5,040    | 3,500            |       |
| Flow-through| 0.60    | 310    | 4,650    | 7,050            |       |
| Wash        |         |        |          |                  |       |
| Eluate      |         |        |          |                  |       |
| Fraction 3  | 0.14    | 6.7    | 48,300   | 345,000          |       |
| Fraction 4  | 0.12    | 5.6    | 78,500   | 654,200          |       |
| Fraction 5  | 0.08    | 5.5    | 34,700   | 434,000          |       |
| Pool (Fractions 3–5) | 0.34 | 17.8 | 171,000  | 503,000          | 48.5  |

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\(\text{AMC release (32). ss-Furin also exhibited a pre-steady-state burst of AMC release in rapidly quenched reactions containing a saturating concentration of Boc-RVRR-MCA (400 $\mu$M) (Fig. 4A). With 10.4 pmol of ss-furin, as determined by quantitative amino acid analysis (data not shown), extrapolation of the linear phase of hydrolysis (500 ms-60 s) revealed a 6.6-pmol burst, indicating that the enzyme in fraction 4 was $\sim 60\%$ active.}

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\(^3\) D. A. Bravo and R. S. Fuller, unpublished observations.
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85-kDa species recognized by the anti-IRβ antibodies (Fig. 5, lane 4).

The difference between the molecular mass of the endogenous β-subunit and the product of cleavage by purified ss-furin could be due to differences in the oligosaccharyl modifications of the two species. To examine this possibility, the N-linked oligosaccharides of the proreceptor, endogenous β-subunit, and ss-furin cleavage product were probed by digestion with N-glycanase (Fig. 5, lanes 6 and 7) and Endo H (Fig. 5, lanes 9 and 10). The proreceptor band was completely sensitive to Endo H, demonstrating that proreceptors were present only in the endoplasmic reticulum or cis-Golgi. The ss-furin cleavage product was also completely sensitive to Endo H, consistent with a precursor-product relationship between this 85-kDa species and the proreceptor. As expected, N-glycanase digestion increased the mobility of both the proreceptor and ss-furin cleavage product by about the same amount as Endo H treatment. In contrast, the endogenous β-subunit band exhibited only slight sensitivity to Endo H digestion (36), indicating extensive modification of N-linked oligosaccharides in the medial Golgi or later compartments. This interpretation was confirmed by observing much more extensive digestion of the endogenous β-subunit band with N-glycanase (37), an enzyme that, unlike Endo H, cleaves oligosaccharides of the complex type.

Effects of Processing Site Mutations on Cleavage of Insulin Proreceptor by ss-Furin—Judging from the heterogeneity of the products, N-glycanase digestion of the endogenous β-subunit appeared to be incomplete, although the most rapidly migrating species approached the mobility of the ss-furin cleavage product. To assess more directly the specificity of the ss-furin cleavage of the solubilized proreceptor, the effects of mutations in the proreceptor processing site were determined.

Substitutions of alanine codons for each arginine codon in the proreceptor cleavage site (sequences and designations in the one-letter amino acid code were: RKRA₉₉, P,Ala; RK₉₉₉₉, P₄Ala) were made in sequences encoding the proreceptor cleavage site. The 85-kDa band is, therefore, authentic hIR⁺11 isoform. Reconstructed cDNAs were subcloned under the control of the constitutive hybrid SV40 early promoter in the mammalian expression vector SRα (38). Mutant and wild type pSRαIR⁺11 plasmids were expressed by transient transfection of COS cells, and detergent-solubilized cell extracts were prepared. The COS cell extracts were incubated at 37°C for 30 min with purified ss-furin (62 units of pooled fractions 3–5) in standard reaction conditions containing either 1 mM CaCl₂ or 10 mM EDTA. As seen previously in the case of human proreceptors in CHO-IR extracts, wild type (Fig. 6A, lane 3) and P,Ala (Fig. 6A, lane 7) proreceptors in the COS cell extracts were cleaved by ss-furin to generate an 85-kDa species recognized by the anti-IRβ antibodies. Cleavage was blocked by Ca²⁺ chelation (compare Fig. 6A lanes 3 and 4 with lanes 7 and 8), consistent with the known Ca²⁺ dependence of Kex2 protease (32) and of furin purified from mammalian cells (17, 18). Under identical assay conditions, ss-furin failed to cleave the P,Ala and P₄Ala proreceptors (Fig. 6A, lanes 5 and 9). This result is consistent with the absolute requirement at furin cleavage sites for Arg at both P₁ and P₄ for processing the hIR⁺11 occurs at the correct processing site. The 85-kDa band is, therefore, authentic hIR β-subunit containing immature N-linked oligosaccharide.

ss-Furin Exhibits the Same Specificity as the Endogenous hIR⁺-Processing Enzyme—Previously, a naturally occurring substitution of Ser for Arg at P₁ in the hIR⁺ processing site was found to block proteolytic maturation, resulting in severe insulin-resistant diabetes in two sibling human patients (11, 12). Subsequent mutational analysis of the processing site demonstrated a requirement for Arg at both P₁ and P₄ for processing in CHO cells (16). In these studies, substitution of Ala for Lys at P₄, or for Arg at P₁ had no effect on cleavage. In the current
experiments, the endogenous COS cell-processing enzyme exhibited similar specificity (Fig. 6B; also -Calcium lanes in Fig. 6A). Wild type hIR+11 and P,Ala mutant proreceptors were cleaved by the cellular enzyme to generate mature 95-kDa \( \beta \)-subunit (Fig. 6B, lanes 1 and 3). However, no mature \( \beta \)-subunit was observed with the P,Ala and P,Ala mutants (Fig. 6B, lanes 2 and 4). A faint 95-kDa band observed in anti-IRP-stained immunoblots of both P,Ala- and P,Ala-transfected cells was also seen in the case of mock-transfected cells (data not shown) and therefore corresponds to endogenous COS cell insulin receptor \( \beta \)-subunit. Thus, the IPR-processing enzyme endogenous to both CHO and COS cells exhibits the same specificity as purified ss-furin does in maturation of hIPR.

It is interesting to note that the level of mature \( \beta \)-subunit seen in cells transfected with the P,Ala mutant was variable and that in the case of all three mutants but not the wild type, an additional proreceptor band was observed at around 200 kDa, just above the major proreceptor band. This species probably corresponds to proreceptor molecules that have undergone extensive modification of N-linked oligosaccharides, suggesting that processing of the P,Ala proreceptor is somewhat delayed in vitro compared with wild type hIPR.

**Comparative Kinetics of Cleavage of Wild Type and Mutant Proreceptors**—To estimate the degree of discrimination by ss-furin between the wild type and mutant proreceptors, the kinetics of cleavage were examined. Fig. 7A demonstrates that with the amount of ss-furin used in the experiment in Fig. 6A (58 units of pooled fractions 3–5), cleavage of wild type hIR–11 proreceptor was actually complete by 5 min of incubation. Incubation with 6-fold less enzyme (9.6 units) resulted in 11% cleavage in 5 min. Therefore, the time course of cleavage for wild type and mutant proreceptors was examined with the lower amount of ss-furin activity. Rates of cleavage were assessed for both the hIR–11 and hIR+11 isoforms of wild type proreceptor. Fig. 7B shows the time course of cleavage of wild type hIR–11 proreceptor by ss-furin at the reduced concentration of enzyme (9.6 units). Densitometric analysis of these data (Fig. 7C) demonstrated a correlation between disappearance of hIR–11 proreceptor and appearance of \( \beta \)-subunit in vitro. The rate of disappearance of the hIR–11 proreceptor appeared to be first order, suggesting that proreceptor concentration was significantly below \( K_m \) for furin cleavage (see "Discussion"). Initial rates of cleavage of the hIR–11 and hIR+11 forms of wild type proreceptors, as measured by the appearance of \( \beta \)-subunit, were approximately the same (Fig. 7D). The rate of cleavage of P,Ala proreceptor was also similar to that of the wild type forms. In contrast, cleavage of the P,Ala and P,Ala proreceptors was undetectable at 60 min, whereas both the wild type hIR–11 and hIR+11 and P,Ala mutant proreceptors were cleaved to completion by that time (Fig. 7D).

Approximately 20% of both the hIR–11 and hIR+11 wild type proreceptors was cleaved by the first time point (3.5 min). Because cleavage of 5% of the P,Ala and P,Ala proreceptors would have been easily detectable at 60 min, the initial rate of cleavage of wild type proreceptors, proportional to \( k_{mon}/K_m \), was at least 70 times that of the P,Ala and P,Ala mutants. Because cleavage of these two mutant proreceptors was undetectable under even more vigorous reaction conditions (i.e. with a 6-fold higher enzyme concentration as shown in Fig. 6A), the difference in \( k_{mon}/K_m \) between the wild type and the P,Ala and P,Ala mutant proreceptors is likely to be considerably greater than 70-fold.

**DISCUSSION**

The results presented here demonstrate that human furin cleaves the human insulin proreceptor at the physiological processing site with the same pattern of specificity as the cellular processing enzyme. First, incubation with ss-furin converts the Endo H-sensitive 190-kDa human insulin proreceptor in cell extracts to an Endo H-sensitive 85-kDa polypeptide that cross-reacts with anti-IRP antisera in immunoblots (Fig. 6). The difference in mobility between the mature, Endo H-resistant 95-kDa \( \beta \)-subunit of hIR and the 85-kDa species produced by ss-furin digestion *in vitro* is accounted for by differences in N-linked oligosaccharide modifications. Second, complete inhibition of cleavage by mutation of either the P, or Arg in the proreceptor processing site demonstrates directly that ss-furin cleaves the proreceptor at the correct site. Moreover, the inhibitory effects of these mutations and the lack of a substantial effect of mutation of the P,Arg to Ala are entirely consistent with the reported specificity both of the cellular insulin proreceptor-processing enzyme and of furin.

This direct biochemical evidence for furin as the physiological insulin proreceptor-processing enzyme is complemented by the analysis of two tissue culture lines found to be defective in maturation of proproteins at furin-like cleavage sites. Moehring and co-workers (39) found that a CHO cell line (RPE.40 cells) with a pleiotropic defect for maturation of precursors of bacterial exotoxins and viral envelope glycoproteins also exhibited a defect for insulin receptor maturation which was corrected by expression of mouse furin cDNA. The Arg-Xaa-Xaa-Arg cleavage motif is conserved in proreceptors structurally related to the insulin proreceptor including the insulin-like growth factor-I receptor, the insulin receptor-related receptor, and the hepatocyte growth factor receptor (6–8). A human colon carcinoma LoVo cell line incapable of processing the hepatocyte growth factor proreceptor was shown to have a point mutation in the *fur* gene (40). This defect was complemented by transfection of LoVo cells with mouse *fur* cDNA (41).

The Endo H sensitivity of the 190-kDa proreceptor in cell extracts indicates that this species is either an endoplasmic reticulum or early Golgi form (Fig. 5, lanes 7 and 10), supporting the conclusion that proreceptor processing ordinarily occurs after modification of N-linked oligosaccharide in the medial and trans-Golgi. This is consistent with localization of furin, at
least when overexpressed, to the trans-Golgi network (24). Cleavage of the endoplasmic reticulum form of insulin proreceptor by purified ss-furin demonstrates that oligosaccharyl modification does not regulate recognition of the cleavage site. Indeed, previous studies showed that an 85-kDa species is produced from the 190-kDa insulin proreceptor when cultured adipocytes are treated with low concentrations of the cation ionophore monensin that block late glycosyl modifications without blocking proreceptor cleavage (42).

ss-Furin cleaved both the hIR-11 and hIR+11 insulin proreceptor isoforms with indistinguishable kinetics (Fig. 7D). Thus, the presence or absence of the 12 residues encoded by exon 11, which lie just upstream from the processing site in hIR+11 proreceptor, does not affect recognition by the protease. In Rat-1 cells transfected with expression constructs of both insulin receptor isoforms, hIR-11 was shown to bind insulin with a 2-fold greater affinity than hIR+11 (43). Thus, the 12 residues encoded by exon 11 may influence the affinity of the α-subunit for insulin, but not the specificity of proreceptor processing.

Engineering of a secreted, soluble form of furin for expression in insect cells was based on a similar approach employed in amplification and purification of ss-Kex2 protease from yeast (32), which has also been used by others to secrete furin from mammalian cells (17, 18). A comparison of the properties of purified ss-Kex2 and ss-furin is instructive, because despite substantial conservation of primary structure (44% identity and 63% similarity in the subtilisin domain), the two enzymes exhibit not only significant similarities but also differences in their catalytic activity and specificity. Both enzymes exhibit selectivity for Arg at P₁, but whereas residues other than Lys or Arg at P₂ substantially reduce the $k_\text{cat}/K_m$ for Kex2 protease (the effect is almost exclusively on $K_m$), substitution of Ala for Arg in the P₂ position of the insulin proreceptor processing site had little effect on processing by ss-furin. Instead, furin appears to exhibit a high degree of specificity for Arg at P₂. Kex2 protease does not exhibit specificity for P₁ substrate residues, although aliphatic residues or methionine tend to be found in this position in natural substrates for the enzyme.

ss-Furin exhibits a substantially lower $k_\text{cat}$ (~0.6 s⁻¹) for Boc-RVRR-MCA than does Kex2 (25 s⁻¹). One possibility is that the tetrapeptide is a poor substrate for furin, in which case furin may require more extensive interactions for an optimal $k_\text{cat}$. A second possibility is that the catalytic cycle of furin is intrinsically slower than that of Kex2. Because the transit time of substrates through the mammalian secretory pathway is nearly 10-fold slower than in yeast, the encounter time for furin with its substrates in vivo is likely to be considerably longer than between Kex2 and its substrates. Thus furin may be as fast as it needs to be.

It is striking that both Kex2 and furin exhibit burst kinetics in cleaving MCA substrates, which most likely means that cleavage of the acylenzyme is rate-limiting. An interesting hypothesis is that the extensive primary structure interactions between Kex2 or furin with substrate peptides somehow favor formation of the acylenzyme over its hydrolysis.

Our direct biochemical studies, in combination with expression studies, provide strong evidence that furin is the cellular enzyme responsible for activation of the insulin proreceptor and most likely the precursors of other members of the IR family of receptors as well. The glycoprotein precursors of numerous lipid-enveloped viruses, including HIV-I 160-kDa glycoprotein and fowl plague virus hemagglutinin, require cleavage by a cellular protease at an Arg-Xaa-Xaa-Arg sites for activation of their fusogenic potential, which is necessary for entry into host cells. Inhibitors of furin activity have been designed (44) and shown to prevent syncitium formation and secondary infection of HIV virions released from cell cultures (26). Efforts that target furin for antiviral drug development must take into account the important cellular roles of the enzyme in the host. For example, although inhibition of insulin proreceptor processing briefly during acute viral infection may be possible, toxicity due to long term inhibition may be unacceptable.

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