been shown to modify the regulatory and/or catalytic subunits of the adenylate cyclase system in rat cortical slices (*20*) and anterior pituitary cells (*21*). In fibroblasts, phospholipids associated with the phosphatidylinositol system act via the inhibitory regulatory protein of adenylate cyclase (*22*). We suggest that two distinct second messenger systems may converge at some level to decrease the same potassium conductance.

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Yeast KEX2 Protease Has the Properties of a Human Proalbumin Converting Enzyme

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Several classes of proteolytic enzymes have been proposed to have a role in the processing of precursor forms of proproteins at paired basic amino acid residues. In higher eukaryotes, a single endopeptidase has yet to fulfill the necessary criteria as the physiologically relevant convertase. The observation of proalbumin circulating in a child with a bleeding disorder caused by an unusual α_1-antitrypsin mutation led to speculation that the presence of this α_1-antitrypsin mutant was inhibitory to the convertase. This provided an additional means of characterizing the processing enzyme. In this study the yeast KEX2 enzyme, a calcium-dependent thiol protease, was found to have all the properties expected for this processing enzyme. KEX2 correctly recognized and cleaved the prosequence in proalbumin. In addition, KEX2 was specifically inhibited by the mutant α_1-antitrypsin but not by other serine protease inhibitors.

PROPROTEIN CLEAVAGE OCCURS AT pairs of basic residues, and cleavage at such sites is an essential feature in the processing of peptide hormones, neuropeptides, and some of the plasma proteins (*1, 2*). A clue to the enzyme involved is provided by the yeast *Saccharomyces cerevisiae*, in which mutations of the *KEX2* gene, coding for a proposed Golgi enzyme, block the processing at Lys-Arg sequences of the secreted yeast α-factor and killer toxin peptides (*3*). Membrane preparations from these yeast mutants have also lost the ability to cleave the dibasic peptide substrate *t*-butyloxycarbonyl -Gln -Arg -Arg -amino -4-methylcoumarin (BOC-Gln-Arg-Arg-MCA). However, reintroduction of the normal *KEX2* gene to the yeast, either on a multicopy plasmid or by integration into the genome, restores the missing proteolytic processing activities (*4*) and the killer expression [Kex^+ (*5*)] phenotype. No such mutation of a converting enzyme has been observed in higher eukaryotes, but several instances of a failure in proprotein cleavage have been recorded in humans. In particular, one unusual and unexplained instance of a failure of proalbumin cleavage occurred in a child who had a reactive center variant of the plasma serine protease inhibitor α_1-antitrypsin (*6, 7*). We report here experiments with the KEX2 enzyme that precisely duplicate the findings in this case. We conclude that the convertase that processes proalbumin to albumin in the liver is likely to be closely related to the KEX2 protease of yeast.

The production of albumin requires, as a final step in the Golgi vesicles (*8*), the cleavage at an Arg-Arg sequence of a six-residue propeptide from proalbumin (*9, 10*). A complete failure of proalbumin processing has been observed in two individuals with variations in this Arg-Arg cleavage site: proalbumin Christchurch (*11*) in which there is Arg-Gln, and proalbumin Lille (*12*) in which there is His-Arg. A third, more unusual, instance of a failure in propeptide cleavage occurred in a child with an abnormal α_1-antitrypsin (*6*), whose plasma contained uncleaved normal proalbumin (*13*) (Fig. 1A). The new variant inhibitor, α_1-antitrypsin Pittsburgh (*7*), had a replacement of its reactive center (358 methionine by arginine), which changed its activity from a general inhibitor of serine proteases to a relatively specific inhibitor of serine proteases that cleave at arginine or lysine residues (*14*). Albumin and α_1-antitrypsin are co-processed in the liver (*15*), and the reasonable conclusion from the observations in the child from Pittsburgh was that the mutant α_1-antitrypsin was acting as an inhibitor of the convertase that cleaves proalbumin (*13*). However, the inference from this, that the proalbumin convertase was a serine protease, ran counter to some evidence that suggested the mammalian propolypeptide or prohormone processing enzymes are thiol enzymes and perhaps cathepsin B–like (*10, 16*).

Together, these experiments of nature provide an exacting set of conditions to be met by a prospective mammalian cell con-

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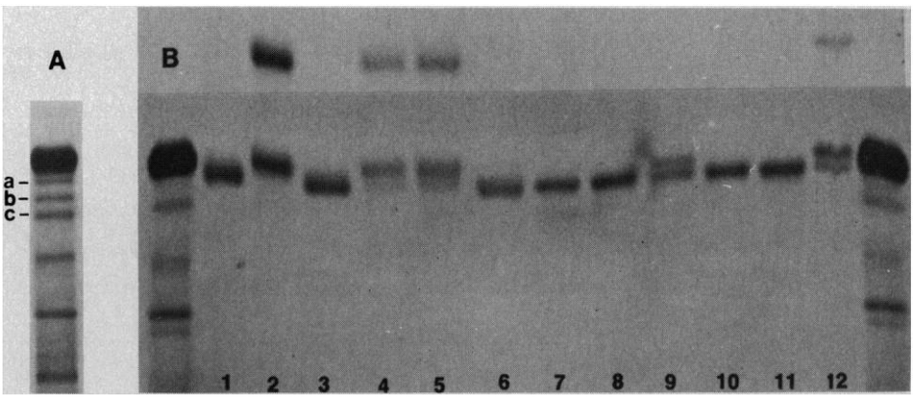

Fig. 1. (**A**) Agarose gel electrophoresis at *p*H 8.6. Plasma of the child from Pittsburgh showing two new abnormal bands: (a) proalbumin (not present in normal plasma); (b) normal α_1-antitrypsin M; and (c) α_1-antitrypsin Pittsburgh (*13*). (**B**) Agarose gel electrophoresis showing specificity and inhibitory properties of KEX2 protease. Lanes 1 and 3, proalbumin; lane 2, proalbumin plus trypsin; lane 4, proalbumin plus KEX2; lane 5, proalbumin plus α_1-antitrypsin and KEX2; lane 6, proalbumin plus α_1-antitrypsin Pittsburgh and KEX2; lane 7, proalbumin Christchurch plus α_1-antitrypsin and trypsin; lanes 8 and 10, proalbumin Christchurch; lane 9, proalbumin Christchurch plus trypsin; lane 11, proalbumin Christchurch plus KEX2; lane 12, markers, albumin plus proalbumin Christchurch. Incubations were performed for 1.5 hours before the addition of $^{63}Ni_I$, in 50 m*M* Hepes and tris, *p*H 7.5, containing 0.25% Triton X-100 and 1 m*M* TPCK, in a final volume of 25 μl at 37°C. Proalbumin and proalbumin Christchurch were each present at 50 μg, trypsin at 0.02 μg, and α_1-antitrypsin and $\alpha_1$antitrypsin Christchurch at 5 μg. The KEX2 preparation was derived from yeast membranes (*3, 4*) and used at a final protein concentration of 4 μg per assay. Serum protein electrophoresis analysis was performed on 4 μl of the assay; the ^{63}Ni autoradiograph is offset above the protein electrophoresis pattern.

vertase. Previous candidates for the convertase are excluded; cathepsin B does not cleave proalbumin (*13*); trypsin cleaves the abnormal (Christchurch) proalbumin as well as normal proalbumin. In lower eukaryotes, the yeast serine protease, convertase Y (*17*), is excluded because it is not a microsomal enzyme and it cleaves between the basic residues. However, as is shown in Fig. 1, the yeast convertase KEX2 meets all these conditions and, furthermore, meets the additional requirement of being inhibited by the variant α_1-antitrypsin Pittsburgh but not by the normal α_1-antitrypsin M.

Conversion of proalbumin to albumin is confirmed by the autoradiograph (see Fig. 1B, offset above) of the complex formed by nickel (^{63}Ni) specifically with the Asp-Ala-His NH_2-terminus of albumin (*18, 19*). Cleavage of normal proalbumin to albumin occurs with both trypsin (lane 2) and KEX2 protease (lane 4) but, unlike trypsin (lane 9), KEX2 protease does not cleave the variant proalbumin Christchurch (lane 11). The fast migrating band generated from proalbumin Christchurch by trypsin (lane 9) does not bind ^{63}Ni because cleavage occurs at the Arg-Gln bond, giving an albumin with an initial sequence of Gln-Asp-Ala-His. A comparison of lanes 7 and 5 shows that normal α_1-antitrypsin M abolishes cleavage of proalbumin by trypsin, but has no effect on cleavage by KEX2 protease. The cleavage of proalbumin by KEX2 protease, however, was completely inhibited by the 358 Met → Arg Pittsburgh variant of α_1-antitrypsin (lane 6). Similar results are shown (Fig. 2) with a plot of KEX2 activity in the presence of increasing concentrations of various plasma serine protease inhibitors. Although the natural α_1-antitrypsin Pittsburgh (358 Met→Arg) and recombinant α_1-antitrypsin Pittsburgh produced in yeast cause complete inhibition, the normal α_1-antitrypsin M causes only partial inhibition, even at concentrations several hundredfold greater than that of the Pittsburgh α_1-antitrypsin. Confirmation that the KEX2 activity was responsible for the cleavage at a pair of basic residues was demonstrated by using identical preparations from a *kex2* strain (*4*). These membranes, from yeast lacking KEX2 activity, were unable to cleave BOC-Gln-Arg-Arg-MCA (*4*). We also found that these membranes were not able to process proalbumin.

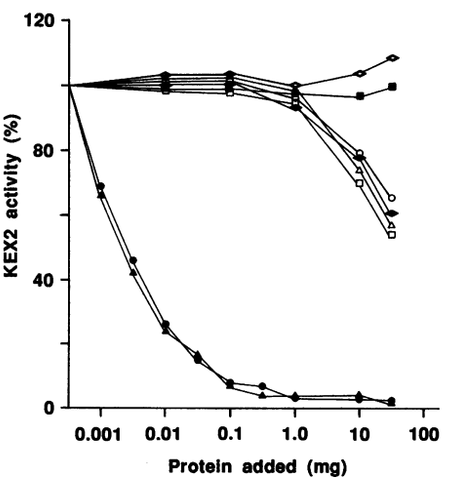

Fig. 2. Inhibition of KEX2 activity by yeast-derived recombinant and human plasma α_1-antitrypsin Pittsburgh (358 Met → Arg). For assay [see (*4*)] we used *t*-butyloxycarbonyl-Gln-Arg-Arg-7-amino-4-methylcoumarin substrate in the presence of 1 m*M* TPCK. Symbols: ○, human plasma α_1-antitrypsin M and ●, human α_1-antitrypsin Pittsburgh, isolated as described (*6*); ◆, yeast recombinant α_1-antitrypsin M and ▲, α_1-antitrypsin Pittsburgh were expressed and isolated essentially as described (*21*); □, antithrombin-III with and △, without heparin; ◇, heparin cofactor II with and ■, without heparin. The excess heparin that interferes with the fluorogenic substrate was removed by Polybrene. The final protein concentration of the KEX2 preparation in each assay was 0.2 μg/ml. Assays were performed in 200 μl for 30 minutes at 37°C. Results are averaged from two independent preparations and are typical of eight experiments.

These results show that the KEX2 protease meets the requirements of the proalbumin convertase. It is a membrane-bound enzyme that cleaves after the normal (Arg-Arg) proalbumin but not the abnormal (Arg-Gln) proalbumin Christchurch. The KEX2 protease also fits the criterion derived from other studies that show that some candidate convertases can be inhibited by thiol reagents (KEX2 is inhibited by iodoacetate, iodoacetamide, Zn^{2+}, Cu^{2+}, Hg^{2+}) but not by specific inhibitors of serine proteases [KEX2 is not inhibited by phenylmethylsulfonyl fluoride, *N*-tosyl-L-lysine chloromethyl ketone, or *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (*4, 20*)]. Finally, the KEX2 protease meets the most exacting requirement of all, in that as a protease not inhibited by classical serine protease inhibitors it is specifically inhibited by the serine protease inhibitor α_1-antitrypsin Pittsburgh. The mechanism of this unexpected inhibition of a thiol protease by a serine protease inhibitor is a puzzle; it is not by the usual formation of a covalent bond, since ^{125}I-labeled α_1-antitrypsin Pittsburgh does not form a high molecular weight complex with the protease when analyzed on SDS polyacrylamide gels. However, the precise duplication of properties in vitro suggests the conclusion that the propeptide cleaving proteases and, in particular, the human proalbumin convertase, are likely to be closely related to the KEX2 protease of yeast.

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Epithelial Wound Healing Enhanced by Transforming Growth Factor–α and Vaccinia Growth Factor

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Epidermal regeneration following middermal injuries to skin requires both proliferation and migration of keratinocytes. Epidermal growth factor (EGF) stimulates the proliferation of keratinocytes in culture, and topical administration of EGF accelerates epidermal regeneration of partial thickness burns or split-thickness incisions in vivo. Transforming growth factor–alpha (TGF-α) and vaccinia growth factor (VGF) have substantial sequence homology with EGF, and all appear to bind to the same receptor protein. Whether TGF-α or VGF can affect epidermal wound healing in vivo is not known. The present studies show that topical administration of TGF-α or VGF in antibiotic cream to partial thickness burns (second degree) accelerated epidermal regeneration in comparison with untreated or vehicle-treated burns. Low levels of both TGF-α and VGF (0.1 microgram per milliliter) appeared to be more effective than EGF in stimulating epidermal regeneration. Regenerated epithelium from burns treated with TGF-α or VGF appeared normal histologically. This finding suggests that topical application of selected growth factors may be useful in accelerating healing of partial thickness injuries.

PEPTIDE GROWTH FACTORS MAY PLAY important roles in the body's response to injury by promoting wound healing. Epidermal growth factor (EGF) present in saliva is thought to accelerate healing of cutaneous injuries in mice as they lick their wounds (*1*). Topical application of EGF accelerated epidermal regeneration of middermal skin injuries (*2*), corneal abrasions (*3*), and increased tensile strength of corneal incisions (*3, 4*). Transforming growth factor–α (TGF-α) (*5*) and vaccinia virus growth factor (VGF) (*6*) have substantial sequence homology to EGF, and all three growth factors appear to bind to and activate a common tyrosine kinase receptor (*7–9*). Whether TGF-α and VGF have activities in vivo similar to those of EGF in promoting wound healing is not known. We now report that TGF-α and VGF both accelerate epidermal regeneration of middermal thermal skin injuries.

Human epidermal keratinocytes express specific high-affinity membrane receptors for EGF (*10*), and EGF increases the lifetime of cultured epidermal cells by stimulating division and minimizing differentiation (*11, 12*). Since regeneration of the epidermal layer after a middermal injury occurs by mitosis and migration of epidermal cells from residual epidermal appendages within the wound and from intact epithelium surrounding the injury (*13*), agents that accelerate mitosis of epidermal cells could accelerate epidermal regeneration of middermal wounds. The effects of TGF-α and VGF on epidermal regeneration in vivo were tested on middermal thermal wounds (second degree burns) on the dorsal thorax of adult pigs. Chemically synthesized rat TGF-α (rTGF-α) or human TGF-α (hTGF-α) (Peninsula Laboratories; Belmont, California) were applied twice a day in a water-miscible antibiotic cream (Silvadene). After 9 days of treatment, the eschar was removed, burns were photographed, and areas of regenerated epithelium were measured by two evaluators using computerized planimetry of enlarged photographs. The methods of treatment were unknown to the evaluators. The three burns treated with different concentrations of rTGF-α (0.1, 1.0, and 10 μg/ml) all showed a substantially larger area of regenerated epithelium than the parallel untreated or vehicle-treated burns (Fig. 1). Similarly, all three burns treated with different concen-

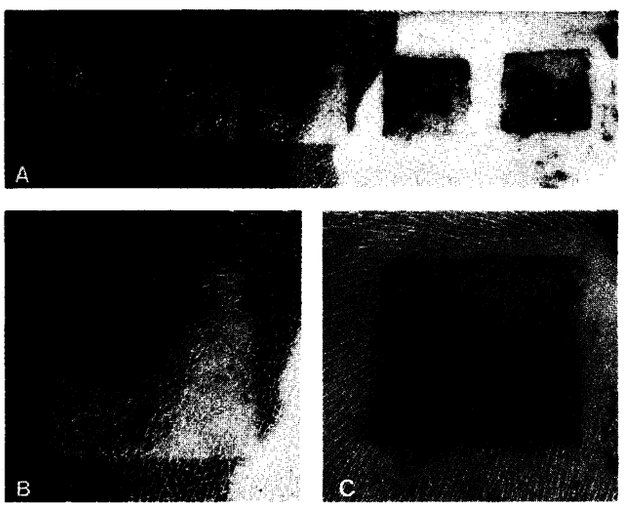

Fig. 1. (**A**) Photograph of middermal thermal wounds (second degree burns), with eschar removed, at 9 days (from left to right) after daily treatment with TGF-α at 10, 1, and 0.1 μg/ml in a water-miscible ointment (Silvadene), no treatment, or treatment with vehicle alone. Enlargements of burns treated with (**B**) TGF-α at 0.1 μg/ml in Silvadene or (**C**) with Silvadene alone.

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