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ROLE OF PROTEIN PROCESSING, INTRACELLULAR TRAFFICKING AND ENDOCYTOSIS IN PRODUCTION OF AND IMMUNITY TO YEAST KILLER TOXIN[1]

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Yeast strains harboring M_1-dsRNA and its packaging virus ScV-L secrete a disulfide-linked, heterodimeric toxin which kills sensitive yeast cells by disrupting plasma membrane function. The mature toxin is derived from a precursor (preprotoxin) which undergoes post-translational processing steps during export via the established yeast secretory pathway. Cleavage by both the *KEX1* and *KEX2* endopeptidases is required for expression of killing activity. The same 1.0 kb open reading frame on M_1-dsRNA directs the expression of immunity to toxin. Differentially processed derivatives of protoxin, as well as protoxin itself, have been proposed to serve as mediators of immunity.

To understand the mechanisms by which the killing and immunity phenotypes can be derived from a common precursor, we have: 1) studied cellular processes implicated in expression of the phenotypes; and 2) developed a system to produce mutants defective in immunity, killing, or both. In the first approach, the role played by both endocytosis and vesicular traffiking in expression of killing and immunity was examined. Strains defective in endocytosis (*end1, end2*) or vacuolar protein localization (*vpl3, vpl6*) were transformed with a plasmid encoding killer toxin under control of the *pho5* promoter. When induced by phosphate starvation, both *end* mutants and all *vpl* mutants expressed killing activity. Immunity to exogenous toxin, however, was significantly decreased in strains carrying both *vpl* mutant alleles and in one of the endocytosis mutants (*end1*)). This suicidal phenotype (*rex* for *r*esistance *ex*pression) has been described previously in M_1-containing strains as a leaky phenocopy. The distinct selective disadvantage of the *rex* phenotype can be overcome in this system by phosphate-mediated repression of killer toxin expression. In the second approach, a strain carrying the phosphate-repressible copy of the toxin gene was mutagenized with EMS, and survivors were scored for the *rex* phenotype. A large percentage of the *rex* mutants isolated also exhibited a *vpl* phenotype, implying that efficacious sorting of vacuolar proteins may be important for expression of immunity. In contrast, the *rex* mutants and the wild type REX^+ strain displayed an END^+ phenotype which was distinct from that of the *end1* and *end2* mutant. At the level of stringency of this fluid phase uptake assay, it appears that defective endocytosis is not a common phenotype obtained among independently isolated and viable *rex* mutants.

INTRODUCTION

The specific mycoviral system we are studying is the type system in *Saccharomyces cerevisiae.* This virus confers upon its host cell the ability to produce an exocellular toxin and specific immunity to this toxin. The existence of virus-like particles in simple eucaryotes is not unusual, and in fact is more the rule rather than the exception (13). The association of these viruses with killer phenomena is, however, less common. Numerous toxin specificity groups exist

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amongst different yeasts and various fungi and progress made in studying these killer systems owes in great part to the ease in scoring the phenotypes of mutants. Cells may have one of four basic phenotypes: killer, K^+R^+; sensitive, K^-R^-; neutral, K^-R^+; or suicidal, K^+R^-. These are readily scored by simple plate assays (16). In this paper we will describe some of our research directed at defining the molecular mechanisms and processes involved in toxin production and in immunity.

The type 1 yeast killer toxin and the determinant for immunity against it are both encoded by a linear 1.9 kb dsRNA molecule termed M_1, found cytoplasmically packaged into icosahedral particles of approximately 40 nm in diameter (3). These virions are comprised of a single, major capsid protein of 88 kilodaltons (KDa), encoded by a separately packaged 4.9 kb dsRNA termed L (12). Toxin acts at the membrane level and is thought to create cation permeable channels in sentitive cells (9). In addition to the *cis* encoded properties of the dsRNA molecules, expression of toxin and immunity depend upon numerous complex interactions with *trans*-acting factors provided by the host (Table 1). These range from factors mediating maturation of the primary translation product of M_1, such as those encoded by the *SEC* (*sec*retion) and *KEX* (*k*iller *ex*pression) genes, to those involved in the process of immunity, either in host-mediated resistance to toxin, encoded by the *KRE* (*k*iller *re*sistance) genes, or in viral-mediated immunity to toxin, encoded by the *VPL* (*v*acuolar *p*rotein *l*ocalization), *END* (*end*ocytosis), and *REX* (*r*esistance *ex*pression) genes. Multiple copies of L and M_1 exist in infected cells at copy numbers of approximately 1000 and 100 respectively. Over 37 additional cellular genes known as *MAK* (*ma*intenance of *k*iller) or *SKI* (*s*uper*ki*ller) genes affect the maintenance of the virus and its replication (23).

TABLE 1. – Genes that interact with the killer system

Gene	Product	Function	Ref
KEX1	carboxypeptidase	toxin processing and activity	5, 10
KEX2	endopeptidase	protoxin processing	5, 14
SEC	multigenic	toxin secretion	5, 15
SKI5	exocellular peptidase	toxin stability	6
MAK	multigenic	dsRNA mainten.	22, 25, 26
SKI	multigenic	dsRNA mainten.	26
KRE1-5	cell wall & memb. comp.	toxin binding	1
REX1	endopeptidase (?)	resistance expression	24
VPL	multigenic	vacuolar protein localization	17
END1	(?)	endocytosis	8

M Encodes a Toxin Precursor

We demontrated several years ago that the primary translation product of the M_1-dsRNA genome is a 35 KDa protein which is a precursor to toxin. This species was detected by *in vitro* translation of denatured dsRNA, and was termed M_1-P1 (3). The *in vivo* product, or preprotoxin, undergoes modification during transit and secretion, following the normal secretion pathways in yeast. The 35 KDa protein is glycosylated in the endoplasmic reticulum to form a 43 KDa protein called protoxin. Protoxin is further processed at a late golgi stage or in secretory vesicles to form two discrete toxin polypeptide subunits, termed α and β (4). These form the dimeric, disulfide-linked mature toxin. The maturation process minimally requires *KEX2*, a dibasic endopeptidase cleaving at the α-β boundary (5) and *KEX1*, a carboxypeptidase removing dibasic C-terminal residues (10). Because M_1 also confers immunity to this toxin, it was hypothesized that the precursor is involved in the immunity process.

Our research has taken two basic approaches to studying toxin production and immunity. One has been to perform saturation mutagenesis of the cloned preprotoxin gene, to define functional regions of the molecule. The other has been to genetically and biochemically analyze mutants in host genes mediating these two processes. The results have lead to the formation of tentative but intriguing models for cytotoxicity and immunity.

Structure of the Preprotoxin Gene and Creation of PTX1

The preprotoxin gene was originally cloned as a complementary DNA in pBR322, using purified transcript from M_1-dsRNA. The nucleotide sequence of the cloned region of M_1, the amino acid sequence at the N-termini of α and β, and the molecular weight of the primary *in vitro* translation product were determined. These were aligned with the sequence at the termini of M_1 to create a model for the organization of the M_1 genome. Figure 1 shows the results of these efforts, illustrating the functional features of preprotoxin: toxin subunit domains, processing sites, glycosylation sites and cysteine residues.

The hydropathy and secondary structure profile (7) of preprotoxin has revealed additional features of the molecule. δ has all the properties of a leader region serving typical functions, but may not be proteolytically removed. α has two hydrophopic regions near its C-terminus separated by a hydrophilic region. We believe this region may constitute a membrane spanning domain of the molecule, forming pores through which protons leak from the cell. β, which may play a role in binding to a cell wall receptor, and γ, which was initially proposed as a domain for immunity function, do not show any strong features on the profile.

Through modification of the yeast *PHO5* gene by mutagenesis, linker insertion and subcloning, a precise

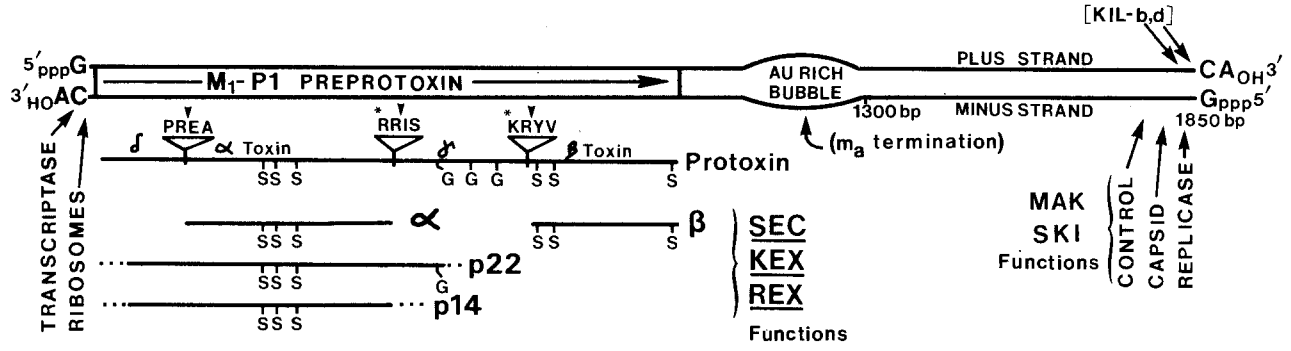

Figure 1. - *Structure of M_1-dsRNA.* A combination of cDNA and RNA sequence analysis predicts the structure shown here. The preprotoxin open reading frame, M_1-P1, is situated at the 5' end of the molecule and encodes a protein with the linear domain structure δ-α-γ-β. Proteolytic cleavage by *KEX2* is denoted by an arrow, *KEX2* cleavage by*, glycosylation by G, and cysteine residues by S. The α, β, p22 and p14 moieties produced from the preprotoxin translation product are shown. The potential positions of *cis*-acting sequences as well as the *trans*-acting counterpart involved in transcription, replication and maintenance of the dsRNA plasmid are also indicated (e.g. *MAK, SKI*).

fusion of the preprotoxin coding sequences to the promoter and terminator of *PHO5* was made. This hybrid allele has been termed *PTX1* (P*HO5*::T*o*X), and has been used to further study preprotoxin expression and maturation. *PHO5* was chosen because it is strongly repressed by inorganic phosphate, and is derepressed more than 1000-fold upon phosphate (Pi) starvation. Figure 2 shows the structure of *PTX1*.

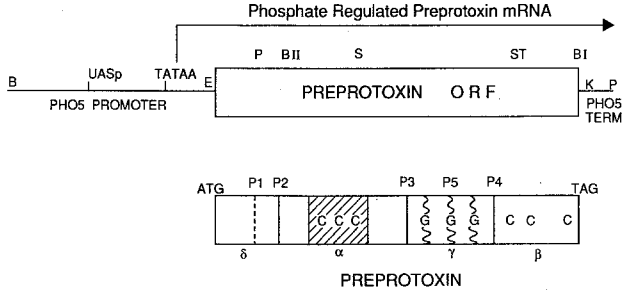

Figure 2. - *PTX1.* The acid phosphatase (*PHO5*) promoter - preprotoxin cDNA gene fusion. Derepression of the *PHO5* promoter by growth on low phosphate medium generates a 1050 bp mRNA directing the production of a 35 KDa preprotoxin (21). Glycosylation (G) and proteolytic cleavage (P2, P3 and P4) lead to the secretion of active toxin subunits α and β bound by disulphide linkages of cysteine residues (C). Additional, unconfirmed proteolytic cleavages may occur at P1 (signal peptide cleavage) and P5 (to release P22 - see text). The shaded area denotes the putative active site of α involved in expression of killer and immunity phenotypes. B, BI, BII, E, K, P, S, and ST denote recognition sites for *BamHI, BglI, BglII, EcoRI, KpnI, PstI, SpeI* and *StuI* restriction endonucleases, respectively.

Model For Immunity

An early model for the mechanism of immunity was formulated based on genetic data (23) wherein mutants of virus-free strains which showed resistance to toxin were isolated. These *kre* mutants fell into two classes: cells with sensitive spheroplasts (*krel, kre2*), and cells with resistant spheroplasts (*kre3*). It was proposed that killing is a 2 stage process in which the toxin interacts with a 1,6 β-D glucan cell wall receptor defined or produced via the action of the *KREI* and *KRE2* genes, and that toxin is thereby brought into contact with a plasma membrane receptor, encoded by the *KRE3* gene, to cause disruption of membrane integrity. The immunity determinant, derived from prepotoxin, may interact with the receptor to prevent its interaction with exogenous toxin.

Genetic analysis of PTX1

To begin to test the models for immunity and processing of preprotoxin, the *PTX1* allele was introduced into a sensitive M_1-dsRNA-free strain (GG100-14D), and transformants were analyzed. These virus-free strains carrying *PTX1* exhibited Pi-regulated expression of the killer phenotype; they were K^-R^- when grown in high phosphate medium, but K^+R^+ when phosphate was limiting. Expression of both phenotypes was under control of the *PHO5* promoter; thus, preprotoxin contains the determinants of toxin and the determinant for specific immunity to toxin. The secreted and intracellular profiles of preprotoxin species in derepressed *PTX1* transformants are the same as those from M_1-dsRNA containing cells. No toxin-related proteins are detected in extracts or cell-free supernatants from phosphate-repressed cells (11).

To further characterize functional domains of protoxin, the *PTX1* allele was mutagenized *in vitro*. This was accomplished by insertion of translation termination sequences, deletion of carboxy-terminal coding regions, and oligonucleotide-directed mutagenesis of specific codons. In each case, the nucleotide sequence was determined, transcripts of the expected size were shown to be produced in a Pi-regulated fashion, products of *in vitro* translation of

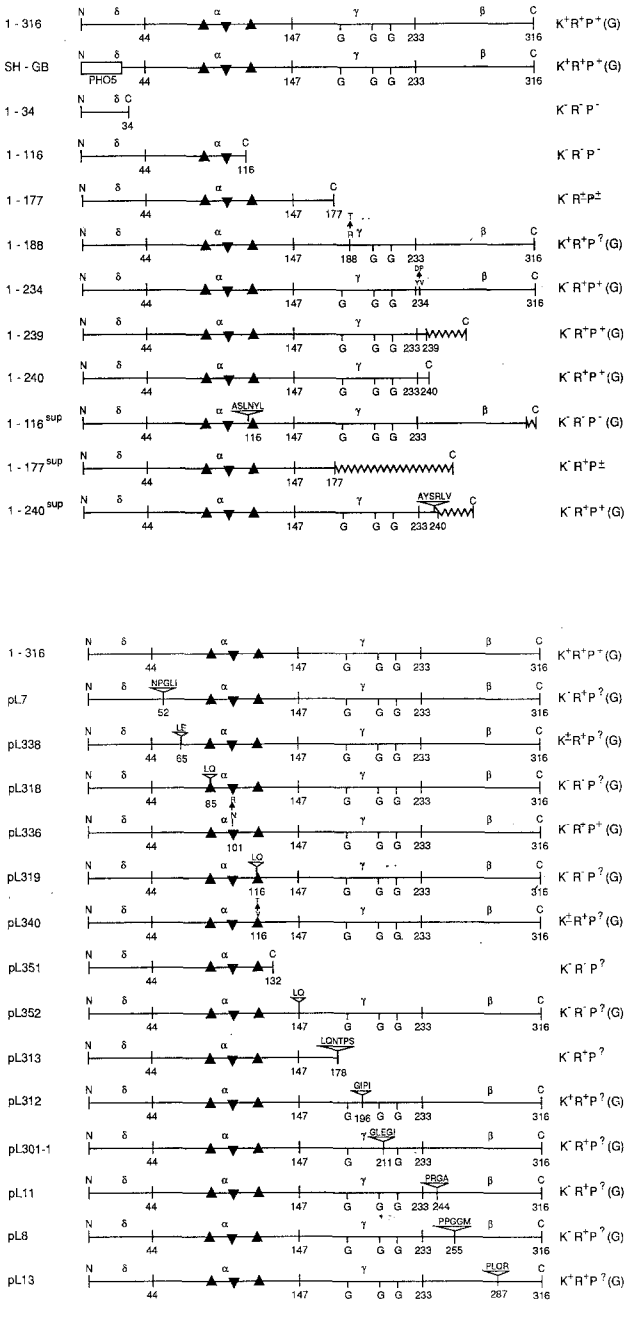

Figure 3. - *Mutagenesis of PTX1.* A series of mutations were introduced into the preprotoxin ORF and expressed in M_1-dsRNA free strains, as described for the *PTX* series (18) and the pL series (2). 1-316 is the 316 amino-acid, wild-type *PTX1* allele possessing the δ-α-γ-β structure with predicted points of glycosylation (G), and proteolytic cleavage , as indicated. The proposed active region of α is indicated by the upright (hydrophobic) and inverted (hydrophilic) triangles. The position of each mutation is indicated by the number of the first residue which differs from the wild-type sequence. Its nature, i.e. insertion or substitution, is denoted by the single letter amino-acid code. Premature truncations were also created. Fusion proteins created in some of the mutations are indicated by a wavy line. The phenotypes conferred are: killing of sensitive strains, K; immunity to exogenous toxin, R; and killing of sensitive spheroplasts, P. G indicates a potential glycosylation site.

these RNAs yielded a protein of the predicted size, and appropriately sized and glycosylated *in vivo* proteins were detected. Each mutant was assayed for the production of intracellular and extracellular toxin-related proteins, for its killer and immunity phenotypes, and for the effect of killer toxin on sensitive spheroplasts (Fig. 3). Three mutations in particular (at residue 239 in β, 116 in α, and 177 in γ) resulted in truncated proteins with revealing phenotypes (18). The *PTX1-239* mutant is defective in killing of whole cells but showed normal immunity. However, a crude preparation of extracellular toxin from this strain kills sensitive spheroplasts. The truncation in *PTX1-116* at residue 116 abolishes both killing and immunity. When *PTX1-116* is introduced into a *SUP7* (suppressor) strain, a secreted protein with a 4 amino acid insertion in the second hydrophobic region of α is produced. This mutant toxin is defective in both killing and immunity, implying that portions of α may play a role in immunity. The *PTX1-177* mutant shows a partial immunity phenotype and weak killing of spheroplasts, suggesting that the N-terminal region of γ, in addition to regions within α, is involved in immunity. This is consistent with a newly observed p22 species playing a role in immunity.

Discovery of the preprotoxin maturation product p22

The predominant toxin related immunoreactive protein in a membrane fraction from *PTX1*-transformed cells or M_1-dsRNA-containing cells was the 43 kDa glycosylated protoxin. In addition to unglycosylated preprotoxin, two species of 11.4 and 9.0 kDa, representing the intracellular counterparts of the α and β processed toxin subunits, were also present. Furthermore, two previosly uncharacterized antigenic species, p14 and p22 (numbers refer to the approximate molecular weights, in kDa) were detected (Fig. 4). The mobility of p14 suggests it is an uncleaved δ-α molecule. Because p22 is glycosylated at a single site and is immunoreactive with antibodies raised against either secreted toxin or a β- galactosidase-γ fusion protein, we suggest that p22 may originate from the N-terminus of protoxin and extend approximately 10 amino acids beyond the truncation in *PTX1-177* (19). Its existence implies an additional processing site in the precursor and a specific protease involved in its cleavage. A most likely candidate is the *REX1* gene product. To test this we analyzed a *rex1* mutant strain for the presence of p22.

Despite the inert nature of the *PTX1-316* allele in strains grown under repressed conditions, a high frequency of reversion (approximately 50%) to toxin resistance was detected in *rex* mutants following derepression of the *PHO5* promoter. Immunoblotting of secreted proteins from *rex* strains indicated near normal levels of wild-type molecular weight toxin subunits. The intracellular toxin profile indicated, in general, wild-type levels of preprotoxin, α, and β, but a marked reduction in the quantities of p22. When toxin

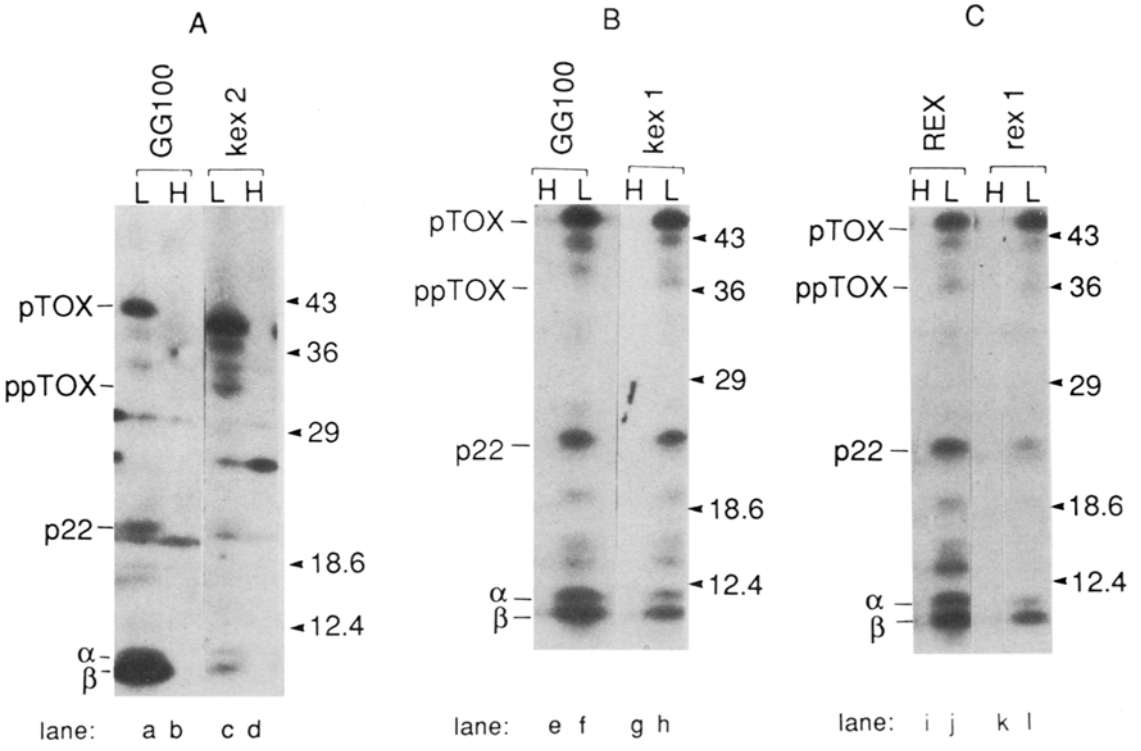

Figure 4. - *Proteolytic processing deficiencies in* kex *and* rex *mutants.* GG100-14D wild-type and congenic *kex2* and *rex1* derivatives were transformed with the *PTX1* allele and grown on high (H) or low (L) phosphate media. Detergent-soluble cell extracts were prepared and analyzed by immunoblotting using anti-toxin IgG and ^{125}I-protein A. *REX* is a spontaneous, immune revertant of the *rex1* mutant strain shown here. pTOX, ppTOX, p22, α and β are as described in the text.

resistant spontaneous *rex1* revertants were analyzed by the same procedures, they displayed normal, or on occasion, elevated levels of p22. These observations are consistent with a role for p22 in the expression of immunity.

Model for Differential Processing of Preprotoxin

We view that processing events leading to production of toxin and immunity could occur by two mechanisms. Protoxin, possibly modified by *REX1*, may be the immunity determinant and bind to the plasma membrane receptor, blocking binding of α delivered from outside or inside the cell. Any excess precursor is then processed by the *KEX1* and *KEX2* gene products to give active α and β. Alternatively, protoxin may be processed by two routes.

One pathway would be mediated by *KEX2* and produce α and β and thus killer toxin. The other pathway (which could be mediated by *REX1* and/or *KEX2* cleavage) would produce a unique immunity determinant, p22.

Support for this model comes from experiments which demonstrated that *KEX* mutants are defective in killing, but not immunity, and *REX* mutants are defective in immunity expression but not killing (23). In Figure 5, these alternate models are depicted schematically.

Chromosomal Genes Involved in Toxin Production and Immunity

In our second approach to understand the immunity process, we isolated chromosomal mutants defective in immunity. Despite the vast array of interactions that the killer system displays with the host cell, remarkably few *trans*-acting factors have been implicated in the expression of immunity to killer toxin. There are two probable reasons for this disproportionality. First, the suicidal nature of such mutations in the presence of active toxin has made isolation of all but leaky representatives virtually impossible. Second, it is possible that the two phenotypes are so closely inter-related that factors mediating immunity but not killing do not exist or are rare. We have addressed the former problem, and the problem of screening for *mak* or *ski* mutants, by utilizing the *PTX1* allele. EMS mutagenesis of an M_1-dsRNA-free strain transformed with this allele, followed by the isolation and subsequent phosphate derepression of the mutant population was performed (Fig. 6). After cultivation of mutagenized cells under repressed conditions (high-Pi medium), single colonies were isolated, derepressed by growth on low-Pi medium and transferred onto a lawn of killer or sensitive cells on low Pi medium. The *rex* mutants (K^+ R-) were detected by their poor growth and normal killing activity on low-Pi medium, while *kex* mutants

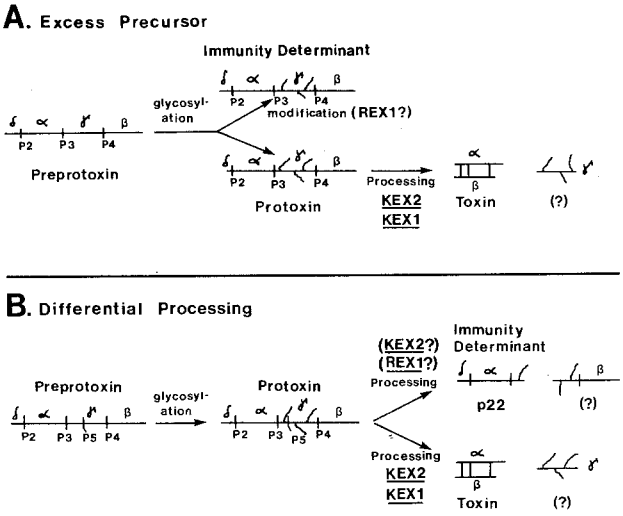

Figure 5. - *A model for preprotoxin maturation.* Two models are proposed for the generation of an immunity determinant from preprotoxin. Model I (excess precursor) proposes that protoxin, possibly modified by *REX1*, serves as the immunity determinant, with the subsequent processing of excess protoxin to produce active α and β. Model II (differential processing) predicts two fates for an intracellular pool of protoxin: *REX1* mediated cleavage gives p22 and an alternate series of proteolytic events mediated by the *KEX* gene products produces the secreted α and β. P2-P5 are potential processing sites using the nomenclature of Sturley *et al.* (18). P1 (not shown) is the predicted leader peptidase recognition site within δ. Glycosylation is denoted by the appendages to the γ domain. Hypothetical disulfide linkage between α and β are indicated by the vertical lines.

(K^-R^+) were detected by their normal growth but reduced or absent killing zones. Non-derepressible (nks) mutants (K^-R^-) were non-killers and poor growers in the presence of the killer tester strain. Panel B of Figure 6 shows the enrichment procedure used for isolation of *kre*-type mutants. The mutant collection was spread onto repressing medium containing active killer toxin or co-cultivated with a constitutive killer followed by auxotrophic selection against the killer strain. Survivors were either constitutive (i.e. *pho*^{c}) killers or non-killer *kre* mutants (K^-R^+).

The mutants obtained are shown in Table 2. A high number of *kre* and *rex* mutants were obtained, suggesting that a substantial multigenic pathway or multiple pathways may be involved non-exclusively in immunity. Since the mutants were not defective in secreting toxin, potential blocks must occur outside of the secretion pathway.

We examined a variety of mutants affecting the modification of secreted proteins: 1) genes affecting a and α factor secretion, 2) genes affecting endocytosis, and 3) genes affecting vacuolar protein localization. Interestingly, the two *vpl* mutants analyzed (*vpl3* and *vpl6*) as well as *end1* but not *end2* were defective in immunity (20). Moreover, a large number of the new *rex* mutants had a *vpl*-like defect, and diverted carboxypeptidase Y to the cell surface (20). When these new *rex* mutants were analyzed for endocytosis, measured by uptake of the dye lucifer yellow into the vacuole, they all appeared normal. All of the new *rex* mutants were nonallelic with *rex1, vpl3, vpl6, end1* and *end2.* The *rex* mutants were further analyzed by immunoblotting for proper secretion and processing of preprotoxin (Fig. 4). As expected from the K^+ phenotype of the *rex* mutants, preprotoxin was apparently processed and secreted normally.

We have begun to consider various explanations for the defective immunity observed in the *vpl* and *end* mutants. Both classes of mutants divert vacuolar proteases to the cell surface, which could degrade the immunity determinant, either during secretion or in the periplasmic space. We believe this is not the case, as *pep4 vpl* double mutants, which fail to process precursors of proteases to mature, active enzymes, also display a K^+R^- phenotype. A more intriguing possibility is the involvement of endocytosis in the cycling of killer receptors from the cell surface to the vacuole. In the presence of the immunity determinant, recycling might not occur and a cell would be immune through desensitization, by virtue of a lack of receptors. In an *end1* mutant, the receptors may stay at the cell surface. Alternatively, receptors may be continuously recycled, and immunity could function by diversion of the killer receptor during transport, in the presence of the immunity determinant, to the vacuole. In a *vpl* mutant the receptor molecules would be routed to the cell surface, leading to a *rex* phenotype. The *pep4 vpl* double mutants would probably have higher levels of stable receptors.

Model for the Mechanism of Killing and Immunity

Figure 7 summarizes our current thoughts on the mechanisms for cytotoxicity and immunity. Killing of a sensitive cell is envisaged as a two stage process

TABLE 2. – Mutant phenotypes obtained

Mutant Class	Phenotype Hi-Pi	Phenotype Lo-Pi	Frequency %
PHOC (dominant)	K^+R^+	K^+R^+	1.00
phoc (recessive)	K^+R^+	K^+R^+	3.00
KRE (dominant)	K^-R^+	K^+R^+	0.17
kre (recessive)	K^-R^+	K^+R^+	0.90
kex (alpha F-)	K^-R^-	K^-R^+	0.03
kex (alpha F+)	K^-R^-	K^-R^+	0.07
nks	K^-R^-	K^-R^-	0.07
rex (vpl)	K^-R^-	K^+R^-	1.80
rex	K^-R^-	K^+R^-	0.94

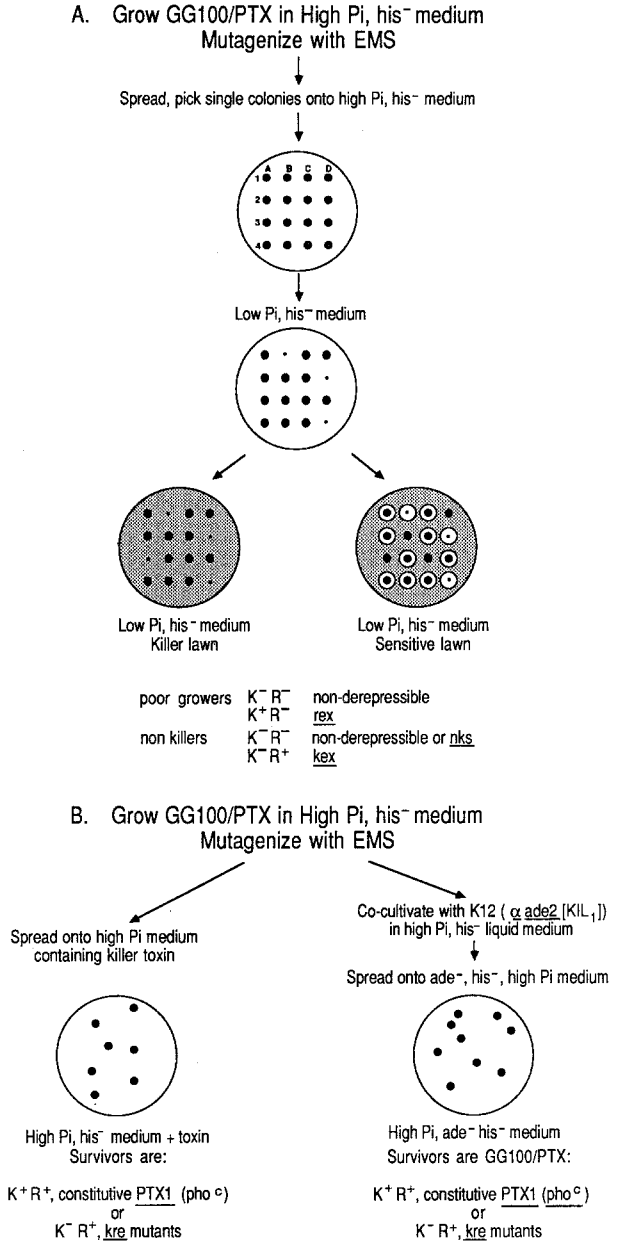

Figure 6. - *Mutagenesis of* trans-*acting factors in expression of the killer system.* An M_1-dsRNA free strain was transformed with the *PTX1* allele and subjected to EMS mutagenesis after growth under repressed conditions (high Pi medium). As shown in Panel A, the single colonies were isolated, derepressed by growth on low phospate media and transferred to low-Pi medium in the presence of a lawn of killer or sensitive cells. *rex* mutants (e.g. colony 2D) were detected by their poor growth and killing activity on low-Pi medium. *kex* mutants (e.g. colony 1D) were detected by their normal growth but reduced or absent killing zones. Non-derepressible (nks mutants, e.g. colony 3A) were non-killers and poor growers in the presence of the killer tester strain. Panel B shows the enrichment procedure used for isolation of *kre*-type mutants. The mutant collection was either spread onto repressing media containing active killer toxin or co-cultivated with a constitutive killer followed by auxotrophic selection against the killer tester. Survivors were either constitutive (i.e. pho°) killers or non-killer *kre* mutants.

involving initial binding of toxin to 1,6 β-D glucan cell wall components (dependent upon the *KRE1, KRE2,* and *KRE5* gene products) via the β domain. The α domain is thus accessible to the *KRE3* plasma membrane receptor (R) where it exerts its lethal effect causing leakage of intracellular metabolites (stage 2). Two alternatives for immunity are represented for an immune cell. Either the immunity determinant (perhaps protoxin or p22) alters or masks the receptor so that it is incapable of interacting with exogenously supplied toxin (the α domain), or the immunity determinant mediates relocation or removal of the receptor from the plasma membrane so that is no longer available for interaction with α delivered from the outside. In the latter model, processes defined by

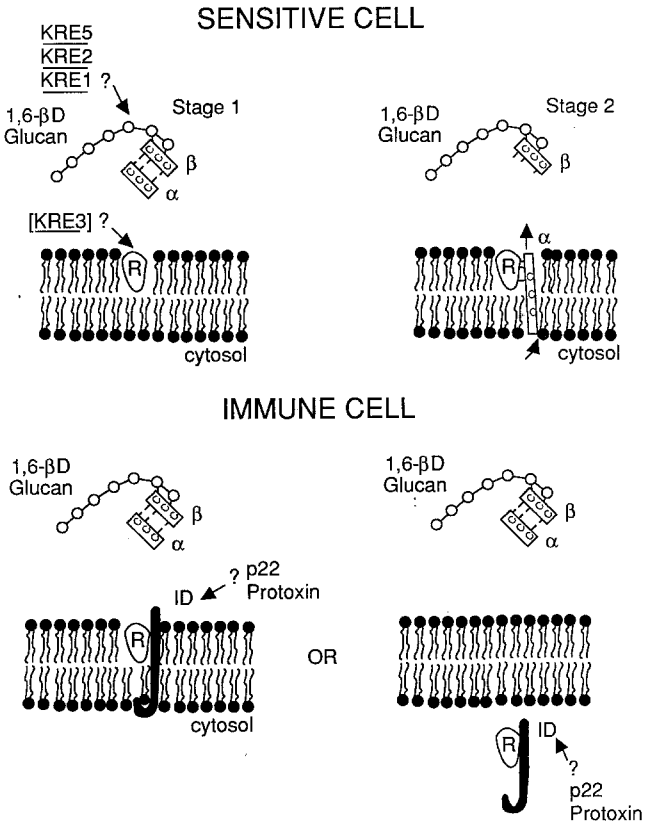

Figure 7. - *Mechanisms of killing and immunity.* Killing of a sensitive cell is envisaged as a two stage process involving initial binding of toxin to 1,6 β-D glucan cell wall components (dependent on the *KRE1, KRE2* and *KRE5* gene products) via the β domain. The α domain is thus accessible to the *KRE3* plasma membrane receptor (R) where it interacts with the membrane to cause leakage of protons (stage 2). Two mechanisms for immunity are represented for an immune cell: either the immunity determinant (perhaps protoxin or p22) alters or masks the receptor so that is incapable of interacting with exogenously supplied toxin (the α domain), or the immunity determinant mediates relocation or removal of the receptor from the plasma membrane so that is no longer available for interaction with α delivered from outside the cell. In the latter model, processes defined by mutations in vacuolar protein localization and endocytosis may mediate certain portions of this pathway.

mutations in vacuolar protein localization and endocytosis genes may mediate certain portions of this pathway. Further research into this fascinating system will provide important insights and parallels for examination of the secretion and trafficking mechanisms of other important but less tractable systems.

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