berg found that protein substrates also cause release of bound ADP (*21*). Repetition of this cycle of activation by nucleotide binding and inactivation through ATP hydrolysis should eventually generate small peptides from large proteins. For each cycle, however, two criteria must be fulfilled by the protein substrate for continued proteolysis: a hydrophobic sequence (*14*) in the active site and occupancy of the regulatory region. Thus, by binding to both sites, a single protein can induce its own destruction, and should not affect the degradation of other proteins (Fig. 2).

The existence of proteases in the cytosol should be highly damaging to the cell unless precise mechanisms or inhibitors exist to limit their activity. Our findings suggest a mechanism by which intracellular protease activity may be controlled. Even if protease La is free in the cytosol, it should be relatively inactive, unless it binds to an appropriate substrate, and denatured proteins seem to be preferred activators of the protease (Fig. 3). This activation mechanism thus may have evolved to increase selectivity and to prevent inappropriate hydrolysis of normal cell proteins. The specificity of protein breakdown in part depends on which proteins bind to and activate protease La. This activation mechanism seems analogous to the regulation of tissue plasminogen activator, which is inactive until it binds to its substrate—a fibrin clot (*22*).

An important further feature of protease La action is its repeated inactivation through ATP hydrolysis, which means that proteolysis ceases after each endoproteolytic round is completed. Thus, the ATPase should help ensure that excessive proteolysis does not occur, after the enzyme has been activated.

This enzyme mechanism thus may represent a kinetic method for isolation of a potentially dangerous enzymatic activity. Presumably, the same selective pressures that favored the sequestration of cellular proteases in lysosomes or hydrolytic vacuoles in eukaryotic cells would have favored careful kinetic regulation of this critical cytosolic protease. These novel regulatory features can probably be found in other enzymes. Like bacteria, mitochondria contain an ATP-dependent pathway for protein breakdown (*23*) and an ATP-hydrolyzing protease that resembles protese La (*6*); it also shows a stimulation of peptidase and ATPase activities by protein substrates (*6*). Perhaps an analogous mechanism involving activation by proteins and inactivation through ATP hydrolysis may also help prevent inappropriate or excessive proteolysis in the ATP-dependent pathways in the mammalian cytosol (*3, 24, 25*).

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Restoration by Calmodulin of a Ca^{2+}-Dependent K^+ Current Missing in a Mutant of *Paramecium*

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A combination of genetics, biochemistry, and biophysics was used to show that calmodulin is involved in the regulation of an ion channel. Calmodulin restored the Ca^{2+}-dependent K^+ current in *pantophobiac*, a mutant in *Paramecium* that lacks this current. The restoration of the current occurred within 2 hours after the injection of 1 picogram of wild-type calmodulin into the mutant. The current remained for approximately 30 hours before the mutant phenotype returned. The injection of calmodulin isolated from *pantophobiac* had no effect. These results imply that calmodulin is required for the function or regulation of the Ca^{2+}-dependent K^+ current in *Paramecium*.

THE EXCITABLE MEMBRANE contains ion channels that control the flow of ions into and out of the cell. The factors that regulate these ion channels are poorly understood. Calcium-dependent K^+ channels are activated by voltage and cytoplasmic Ca^{2+}, and they exert a control on various Ca^{2+}-regulated functions of the cells through membrane repolarization (*1, 2*). The molecular mechanisms by which this channel is regulated are unknown. Single-channel conductance and toxin sensitivity distinguish several types of Ca^{2+}-dependent K^+ channels (*3*). A Ca^{2+}-dependent K^+ current of the unicellular *Paramecium tetraurelia* has been studied macroscopically by means of a whole-cell voltage-clamp technique (*4, 5*) and microscopically in inside-out patches excised from surface membrane (*6*). The macroscopic Ca^{2+}-dependent K^+ current in *Paramecium* is almost entirely missing in a class of mutants called *pantophobiac* (*5*). Since this current participates in shutting off the Ca^{2+} excitation, its loss prolongs membrane excitation and exaggerates locomotor responses to stimuli (*5*). We showed earlier that the behavioral defect of two of these mutants (*pntA* and *pntB*) can be corrected by the microinjection of cytoplasm from wild-type *Paramecium* (*7*). We now report that the factor that restores the Ca^{2+}-dependent K^+ current is calmodulin.

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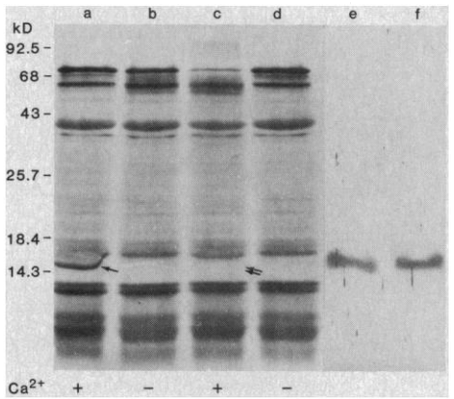

Fig. 1. SDS-PAGE of the heated high-speed supernatant fraction (S_3) and calmodulin. The S_3 fractions and calmodulin were prepared as described in Table 1. Protein (40 μg) was added to lanes a to d and 2 μg was added to lanes e and f. The samples were run on a 15% SDS-PAGE with the buffer system described by Laemmli (*20*). (Lanes a and b) S_3 fractions from the wild type; the arrow shows the calmodulin band that has a greater mobility in the presence of Ca^{2+}. (Lanes c and d) S_3 fractions from the wild type prepared in the presence of 5 m*M* EGTA; the double arrow indicates the lack of calmodulin. (Lane e) Calmodulin from wild-type *Paramecium*. (Lane f) Calmodulin from *pntA*. Lanes a, e, and f had 10 m*M* $CaCl_2$ added to the sample buffer, lane c had 30 m*M* $CaCl_2$ added to overcome the EGTA added while preparing the sample, and lanes b and d had 10 m*M* EGTA added. Lanes a to d were stained with Coomassie blue, and lanes e and f were silver-stained. The left lane shows the positions of the molecular weight standards.

This finding indicates that calmodulin participates in the function or control of the Ca^{2+}-dependent K^+ current of *Paramecium*.

When approximately 300 pg (20 pl) of a supernatant fraction of wild-type cytoplasm obtained by high-speed centrifugation (wild-type S_2; see legend to Table 1) was injected into *pntA* cells, the wild-type phenotype was partially restored, as judged by the behavioral response of the cells in a solution containing 10 m*M* Na^+ and 5 m*M* tetraethylammonium chloride (Table 1). Three hundred picograms of the S_2 fraction is the equivalent of that obtained from one or two cells. No such restoration was observed when *pntA* S_2 was used. If the wild-type S_2 fraction was heated at 95°C for 5 minutes, the supernatant fraction (the S_3 fraction) injected into *pntA* still resulted in a return to a near wild-type phenotype (Table 1). The restoration was temporary; without cell growth and division, the mutant phenotype returned within 30 hours.

When the proteins of the S_3 fraction were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), there was a prominent band at 16.5 kD that had an increased mobility when Ca^{2+} was added to the sample buffer (Fig. 1, lanes a and b). This same band was present in the S_2 fraction but was such a minor component in the mixture that it was barely discernible. Since calmodulin has a molecular weight and mobility shift in the presence of Ca^{2+} similar to those indicated by this band (*8*), the phosphodiesterase (PDE)-stimulating activity of the S_2 and S_3 fractions was determined [calmodulin stimulates PDE activity (*8*)]. Both fractions contained PDE-stimulating activity (Table 1). We therefore concluded that the fractions active in restoring *pntA* contain calmodulin.

Because the K^+ current decreased in *pntA* is Ca^{2+}-activated, the factor that restores *pntA* is heat-stable, and there is calmodulin in the fractions that restore *pntA* to the wild-type phenotype, we isolated calmodulin from wild-type cells to test its ability to restore the *pntA* cells. The calmodulin was purified to homogeneity, as judged from SDS-PAGE gels with silver staining (Fig. 1, lane e). The calmodulin had normal PDE-stimulating activity (Table 1). When this fraction was injected into the *pntA* cells, there was almost complete restoration of the wild-type phenotype (Table 1). The restoration began within 15 minutes, reached a maximum at 2 hours, lasted for over 20 hours, and was lost by 30 hours (Fig. 2). The control (the injection of calmodulin isolated from the *pntA* cells) did not restore the wild-type phenotype. The *pntA* calmodulin, purified to homogeneity (Fig. 1, lane f), did have PDE-stimulating activity, however (Table 1). When calmodulin isolated from *Dictyostelium* or bovine testes was injected, no such restoration was seen (Table 1). Therefore, the ability to cure *pntA* was not common to all species of calmodulin. Since there are at least 11 amino acid differences between ciliate and mammalian calmodulin (*8*), this result was not surprising.

From the above results, we predicted that removing calmodulin from the wild-type S_2 fraction would eliminate its ability to restore the wild-type phenotype to the *pntA* cells. When the S_2 fraction was heated at 95°C for 5 minutes in the presence of excess EGTA, the calmodulin band at 16.5 kD was missing (Fig. 1, lanes c and d), as was the PDE-simulating activity (Table 1). This was expected because calmodulin is denatured and precipitates when heated in a Ca^{2+}-free environment (*9*). When this fraction was injected into *pntA*, no restoration of the wild-type phenotype was seen (Table 1). Therefore, removing the calmodulin was correlated with an elimination of the ability to restore *pntA*.

As stated earlier, the pantophobiac mu-

Table 1. Restoration of the wild-type phenotype in *pntA* by cytoplasmic fractions. The S_2 fractions (high-speed cytoplasmic supernatant) were prepared as described previously (*17*). The S_3 fractions were prepared by heating the wild-type S_2 (in the presence of 5 m*M* $CaCl_2$ or 5 m*M* EGTA) for 5 minutes in a boiling water bath. The heated material was centrifuged in a microfuge for 4 minutes, and the resulting supernatant was the S_3 fraction. The *Paramecium* calmodulin was prepared by taking the pelleted material from the cell homogenization procedure and sonicating it in the presence of 1 m*M* EGTA. This procedure was repeated three times, and the supernatants were pooled and centrifuged at 100,000 rev/min for 60 minutes. The supernatant was concentrated and further purified on a DEAE-cellulose anion exchange column. The fractions containing calmodulin (as determined by PDE-stimulating activity) were pooled, Ca^{2+} was added to attain a concentration of >1 m*M*, and further purified using phenyl-Sepharose column chromatography. The calmodulin was eluted with 5 m*M* EGTA. The cells were pressure-injected with 20 pl (approximately 10% of the cell volume) of material, with previously described techniques (*17*). The injected cells were incubated in a buffer solution of 4 m*M* KCl, 1 m*M* $CaCl_2$, 1 m*M* Hepes, and 0.01 m*M* EDTA (*p*H 7.2) plus 10% culture medium depleted of bacteria; this solution prevented the cells from undergoing growth and division. The cells were tested for their ability to swim backward in a solution of 10 m*M* NaCl and 5 m*M* tetraethylammonium chloride added to the incubation solution. The percentage of the wild-type phenotype was calculated as $100[1 - (X - t_{wt})/(t_{pntA} - t_{wt})]$, where X is the time (seconds) of backward swimming of the injected cells and t_{wt} and t_{pntA} are the control times of wild-type and *pntA* cells. There were six cells injected for each sample, with the average given (± SD). The PDE-stimulating assay was a modification of that used by Rauh and Nelson (*18*). PDE-stimulating activity is expressed as a multiple of the prestimulation value. Inorganic phosphate released by the reaction was measured by the method of Chen *et al.* (*19*). The maximum stimulation was that seen above the basal level when no calmodulin was added and corresponded to a specific activity of 25 nmol of inorganic phosphate per minute per milligram. The experiment was done twice for each sample, and both values are listed.

Source of injected material	Percent of wild-type phenotype	PDE-stimulating activity
S_2 (wild type)	74 ± 4	1.9, 1.8
S_2 (*pntA*)	0	2.3, 2.2
S_3 (+Ca^{2+})	88 ± 5	2.0, 1.8
S_3 (+EGTA)	0	0, 0
Calmodulin (wild type)	98 ± 2	2.2, 2.6
Calmodulin (*pntA*)	0	1.6, 1.8
Calmodulin (*Dictyostelium*)	0	2.1, 2.2
Calmodulin (bovine)	0	2.5, 1.9

tants have a greatly decreased Ca^{2+}-dependent K^+ current. In order to determine whether the *pntA* cells whose behavior is restored by the calmodulin have a similarly restored current, we used a voltage-clamp method to study the injected cells showing the restored wild-type behavior. The wild-type cells had a steadily increasing outward current during the depolarization step (Fig. 3A, arrow), with an outward tail current when the voltage was returned to the resting level (double arrow). In contrast, *pntA* had a declining outward current during the depolarization step (Fig. 3B, arrow), with little or no tail current (double arrow). When the *pntA* cells were injected with calmodulin from wild-type cells and studied 4 hours later, there was an obvious return of the outward current (Fig. 3C), whereas the control cells injected with calmodulin from *pntA* showed no such restoration (Fig. 3D). When the other ion currents of *Paramecium* (for example, the Ca^{2+} inward current and votlage-dependent K^+ outward current) were observed, they appeared normal before and after the injection of calmodulin. Therefore, the behavioral restoration seen after calmodulin injection (Fig. 2) correlates with a return of the Ca^{2+}-dependent K^+ current missing in the *pntA* cells.

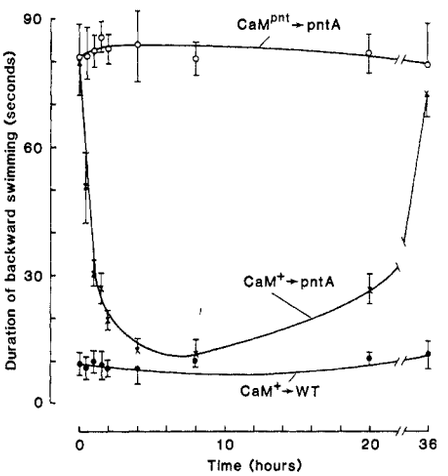

Fig. 2. The time course for the restoration of *pntA* after injection of calmodulin from wild-type *Paramecium* (CaM^+). The cells were injected as described earlier; 1 pg of calmodulin isolated from wild-type *Paramecium* (as described in Table 1) was injected into each cell at time 0; 1 pg of calmodulin corresponds approximately to the amount contained in one cell. The injected cells were placed in a medium that prevented growth and division at 28°C. At each time point, the cells were tested for their duration of backward swimming in a solution containing 10 m*M* Na^+ and 5 m*M* tetraethylammonium, then returned to the resting solution. The control *pntA* cells were injected with calmodulin prepared from *pntA* cells (CaM^{pnt}); the control wild-type cells (WT) were injected with the buffer solution (10 m*M* tris-Cl and 1.5 m*M* EGTA at *p*H 7.2) in which the calmodulin was prepared. Each point represents four cells (± SD).

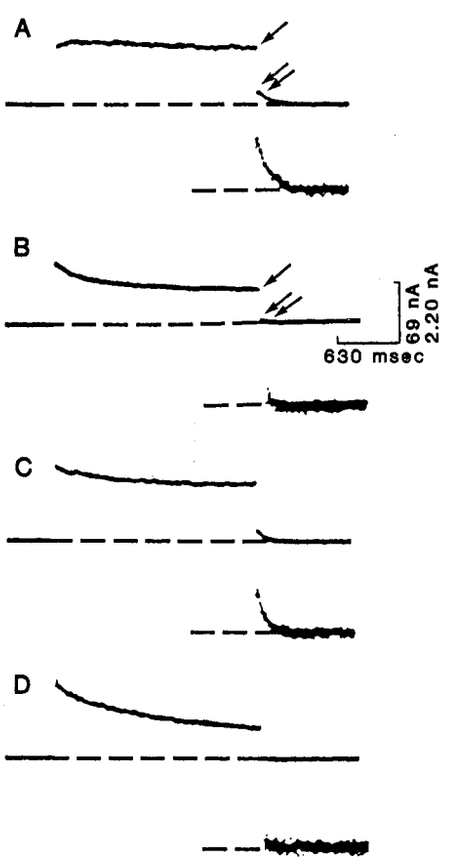

Fig. 3. Voltage-clamp analysis of the Ca^{2+}-dependent K^+ current in *Paramecium* cells injected with calmodulin or buffer. The methods of voltage clamp are described in (*5*). The electrodes contained 3*M* KCl, and the cells were bathed in 1 m*M* KCl, 1 m*M* $CaCl_2$, 1 m*M* MOPS (morpholinopropanesulfonic acid), and 10^{-2} m*M* EDTA (*p*H 7.2). A depolarization step was given from the resting level of −40 mV to −5 mV and held for 2 seconds before returning to −40 mV. The cells were microinjected with either 20 pl of buffer solution [4 m*M* KCl, 1 m*M* $CaCl_2$, 1 m*M* Hepes (*N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid), and 10^{-2} m*M* EDTA at *p*H 7.2] or calmodulin (1 pg) 4 hours before the voltage-clamp analysis was performed. In (A) to (D), the inset shows the tail current amplified four times. (A) Wild-type cells injected with 20 pl of buffer solution showed the Ca^{2+}-dependent K^+ current. The single arrow indicates the late outward current upon depolarization, primarily through the Ca^{2+}-dependent K^+ channel (*5*). Upon repolarization, there was an obvious tail current (double arrows) that is expanded in the inset for clarity. (B) The *pntA* mutant, injected with 20 pl of buffer solution, virtually devoid of the Ca^{2+}-dependent K^+ current. There was a declining late outward current (arrow) upon depolarization, which indicates a nearly complete loss of the Ca^{2+}-dependent K^+ current (*5*). The drooping outward current during the depolarization is largely the leakage current and the voltage-dependent K^+ current (*4*). There is little or no tail current upon repolarization (double arrows and inset). (C) The *pntA* mutant after the injection of wild-type calmodulin (see text) showed a return of both the late outward current and the tail current. (D) The *pntA* mutant injected with *pntA* calmodulin showed no restoration. There was virtually no Ca^{2+}-dependent K^+ current, as indicated by the lack of both the late outward current and the tail current.

Our results indicate a role for calmodulin in the regulation of the Ca^{2+}-dependent K^+ current. There is no understanding at the present time of the chemistry of this channel—for example, the nature of the domain that binds the Ca^{2+}. Calmodulin may be involved in this process as the Ca^{2+}-sensing element of the channel. Alternatively, calmodulin could stimulate a Ca^{2+}-calmodulin–dependent protein kinase that in turn phosphorylates the channel. There have been reports on the effects of adenosine 3′,5′-monophosphate–dependent phosphorylation on Ca^{2+}-dependent K^+ currents (*10, 11*). This modification could also play a role in the maturation of the channel [such as developing Ca^{2+} sensitivity (*12*)] or in removing inactivation of the channel. Studies in which calmodulin antagonists were used suggest a role for calmodulin in the regulation of the Ca^{2+}-dependent K^+ current of red blood cells (*13*) and sarcoplasmic reticulum (*14*). However, these studies are indirect, and the interpretation is complicated because calmodulin inhibitors have multiple effects on cells (*15, 16*). Yet activities of single Ca^{2+}-dependent K^+ channels in excised patches, including those of *Paramecium*, argue against a direct role for soluble calmodulin in these channels. The apparently normal function of the recorded channels will have to be explained by the indirect action of calmodulin or a bound form of calmodulin retained after excision.

Our results do not necessarily imply that the single, recessive lesion on *pntA* resides within the calmodulin molecule itself. Calmodulin may mimic another molecule (possibly a calmodulin-like molecule) whose function *pntA* is lacking, or *pntA* may code for a molecule that modifies the calmodulin so it can regulate the Ca^{2+}-dependent K^+ current. Knowledge of the properties of the calmodulin from *pntA* may indicate whether there is an alteration in the structure of the calmodulin molecule.

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Induction of Macrophage Tumoricidal Activity by Granulocyte-Macrophage Colony-Stimulating Factor

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Monocytes are a subpopulation of peripheral blood leukocytes, which when appropriately activated by the regulatory hormones of the immune system, are capable of becoming macrophages—potent effector cells for immune response to tumors and parasites. A complementary DNA for the T lymphocyte–derived lymphokine, granulocyte-macrophage colony-stimulating factor (GM-CSF), has been cloned, and recombinant GM-CSF protein has been expressed in yeast and purified to homogeneity. This purified human recombinant GM-CSF stimulated peripheral blood monocytes in vitro to become cytotoxic for the malignant melanoma cell line A375. Another T cell–derived lymphokine, γ-interferon (IFN-γ), also stimulated peripheral blood monocytes to become tumoricidal against this malignant cell line. When IFN-γ activates monocytes to become tumoricidal, additional stimulation by exogenously added lipopolysaccharide is required. No such exogenous signals were required for the activation of monocytes by GM-CSF.

MACROPHAGES ARE A HETEROGENEOUS population of cells involved in many aspects of immunity. Macrophages are required accessory cells in the development of specific immune responses mediated by T and B cells [reviewed in (*1*)]. In addition, macrophages themselves mediate nonspecific effector functions in the resistance to and eradication of microorganisms and neoplastic disease (*2*). The expression of these functions in vitro requires stimulation of monocytes or macrophages with an activating agent (*3, 4*). Substances capable of activating monocytes and macrophages include bacterial products such as lipopolysaccharide (LPS) or peptidoglycan (*3*) and, in addition, lymphokines secreted by activated T lymphocytes (*4*). Recent studies of such T cell–derived lymphokines have focused primarily on γ-interferon (IFN-γ). Treatment of macrophages in vitro with IFN-γ results in increased microbicidal activity, expression of cell surface antigens, and increased secretory activity (*5*). IFN-γ stimulates nonspecific tumoricidal activity of macrophages when LPS is present as an additional signal. Thus IFN-γ acts to prime the macrophage, making it more sensitive to the triggering effects of suboptimal amounts of LPS (*6*).

Whether molecules other than IFN-γ have the capacity to induce tumoricidal activity in macrophages has not been determined. Analysis of crude supernatants of cloned T cells or T-cell hybridomas (*7*) has generally failed to separate IFN-γ from any other lymphokines that activate macrophages to become tumoricidal. Although studies with antisera and monoclonal antibodies to IFN-γ have confirmed that IFN-γ activates macrophages, the existence of additional macrophage-activating lymphokines was not shown (*8*). However, investigators using biochemical fractionation of lymphokine preparations have been able to demonstrate the stimulation of macrophages to become tumoricidal by partially purified fractions devoid of detectable IFN (*9*).

Recombinant DNA technology combined with cellular bioassays in vitro has led to the cloning of the complementary DNA (cDNA) of several lymphokines, including IFN-γ (*10*). The availability of recombinant IFN-γ has confirmed the previous reports of the macrophage-activating properties of this lymphokine and the requirement for a second signal, such as LPS, to induce nonspecific tumoricidal activity (*8, 11*).

Recently, we (*12*) and others (*13*) reported cloning a cDNA for the human T cell–derived lymphokine granulocyte-macrophage colony-stimulating factor (GM-CSF). We report that purified recombinant human GM-CSF was capable of stimulating human peripheral blood monocytes to become tumoricidal against the human malignant melanoma cell line A375. GM-CSF cDNA was

Table 1. Differential requirement for LPS triggering of macrophage tumoricidal activity induced by GM-CSF and IFN-γ. Purified peripheral blood monocytes were prepared and cultured as described in Fig. 2 in the presence of lipopolysaccharide (LPS-W, Difco Laboratories), purified natural IFN-γ (Meloy Laboratories) or purified recombinant GM-CSF. After 24 hours, the culture medium was replaced, and [^{125}I]iododeoxyuridine-labeled A375 target cells were added. After an additional 72 hours, residual adherent A375 cells were harvested and counted. Units of IFN-γ are determined with a virus plaque reduction assay and comparison with an NIH international IFN-γ standard. GM-CSF units were determined as described by Cantrell *et al.* (*12*).

Lymphokine	Percent cytotoxicity with LPS (ng/ml) at				
	0	10.0	1.0	0.1	0.01
None	0	84	83	0	0
IFN-γ					
100 U/ml	0			40	
10 U/ml	0			28	
1 U/ml	0			25	
GM-CSF (500 CFU-C/ml)	63				

Immunex Corporation, 51 University Street, Seattle, WA 98101.