Independent Regulation of Two Cytoplasmic Processing Stages of the Intermediate Filament-associated Protein Filaggrin and Role of Ca$^{2+}$ in the Second Stage*

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One of the final events in cornification of epidermal cells is processing of profilaggrin to the keratin-associated protein filaggrin. Processing involves several proteolytic events and occurs in two discrete proteolytic stages (Resing, K. A., Walsh, K. A., and Dale, B. A. (1984) J. Cell Biol. 99, 1372–1378; Resing, K. A., Walsh, K. A., Haugen-Scofield, J., and Dale, B. A. (1989) J. Biol. Chem. 264, 1837–1846). In a keratinocyte cell line derived from newborn rat epidermis, these two stages are independently regulated. Profilaggrin was expressed when the cells reached confluence; processing to intermediates began 24–36 h later (stage one), with filaggrin appearing at 48 h (stage two). Stage two processing required calcium in the medium with maximum processing occurring at 5–10 mM. Furthermore, stage two processing was inhibited by nifedipine, a calcium channel blocker, suggesting that calcium influx activates this event. Second-stage processing was also inhibited by the protease inhibitor leupeptin, implicating calpain. Confluent cells had higher levels of calpain I than subconfluent cells; in confluent cells, two immunoreactive bands were detected, comigrating with inactive (80 kDa) and activated (78 kDa) calpain I. In cells processing profilaggrin, most of the calpain I was in the 78-kDa form, implying extensive activation, supporting a role for calpain in processing.

Differentiation of epidermal keratinocytes is a multistep process ultimately leading to the formation of squamous cells which have cornified cell envelopes and are filled with an ordered array of densely packed intermediate filaments embedded in a matrix (see Ref. 1 for review of epidermal differentiation). The rearrangement of intermediate filaments involves filaggrin, a histidine-rich, basic protein (28 kDa in mouse, 42 kDa in rat) which is synthesized as a high molecular weight precursor called profilaggrin (see Ref. 2 for review). Profilaggrin does not aggregate with keratin filaments (3, 4), and the release of filaggrin must be carefully controlled to avoid premature keratin aggregation. It is highly phosphorylated (5–7), contains 14 or more domains of filaggrin connected by linker segments (8, 9), and is deposited in non-membrane-bound, cytoplasmic keratohyalin granules (10). The processing of profilaggrin to filaggrin is a potentially useful model system for understanding regulated cytoplasmic processing of proteins, about which little is known.

In vivo pulse labeling of newborn mouse or adult guinea pig epidermis with [3H]histidine has shown that processing occurs in two distinct stages (11, 12). These stages are separated in time and produce intermediates that consist of several domains of filaggrin, although the timing of the stages and the size of the intermediates varies. Intermediates are also observed in human (13), rat and rabbit (14) epidermis, suggesting a common processing mechanism in all mammals. Processing involves complete dephosphorylation and removal of the linker segments by proteolysis. Phosphatase 2A has recently been implicated in dephosphorylation of profilaggrin (15); phosphatase 2A removes a limited subset of phosphate moieties, suggesting that at least one more phosphatase is involved. It has been proposed that the two-stage processing involves separate cleavage of two different types of linker segments by two different proteases, and that each primary cleavage event is followed by trimming of the linker remnants (9). Thus each of the two stages of processing consists of at least two distinct proteolytic steps.

The proteolytic processing is best characterized in mouse profilaggrin where the two linker segments have different primary structures (9, 16). The two types are the α$_2$ segments containing a Gly–Phe sequence and the α$_3$ segments containing a Gly–Tyr sequence; the intermediates contain only α$_3$ segments, implying that the first-stage protease cleaves only α$_3$ segments. Limited proteolysis of profilaggrin (9) and restriction mapping of the cDNA (16) have demonstrated that the two types of linker segments are randomly distributed throughout the profilaggrin, so that cleavage at the α$_3$ linker would yield the expected multi-domain intermediates. This model is supported by the demonstration of an epidermal protease that cleaves profilaggrin into multi-domain intermediates; it fits the characteristics expected of a first-stage protease (9), although little is known about its regulation.

The α$_2$ and α$_3$ proteases are potential targets for regulatory action that would produce the two-stage processing. In this study, the second-stage processing is shown to respond to external Ca$^{2+}$. The role of Ca$^{2+}$ in profilaggrin processing gives insight into the regulation of the later stages of the formation of the stratum corneum and may provide a mechanism for coordinating filaggrin release with other known Ca$^{2+}$-depend-

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ent events in the terminal differentiation of keratinocytes. Recent studies show that the N-terminus of profilaggrin is a Ca²⁺-binding domain (17, 18). Although the physiological function of this domain is not understood, its presence within profilaggrin lends support to the significance of Ca²⁺ in the process of cornification.

MATERIALS AND METHODS

Cell Culture Methods—A rat keratinocyte cell line derived from newborn rat epidermis (19) was generously provided by Drs. Kubilus and Baden (Department of Dermatology, Massachusetts General Hospital, Boston, MA). Cells were seeded in six-well plates (Corning Glass Works, Corning, NY) at 1.4 × 10⁵ cells/cm² in Dulbecco's modified Eagle's medium (DMEM; low glucose, Life Technologies, Inc.) with 10% fetal calf serum (HyClone, Logan UT) (DMEM:FCS) containing 0.4 μg/ml hydrocortisone (Sigma), 100 units of penicillin/100 μg of streptomycin/ml (Penstrep, Life Technologies, Inc.) and 5% CO₂. Cultures were fed 3 or 4 days after plating; the cells became confluent after 6 days, as judged by phase microscopy. Pharmacological agents were mixed into fresh medium at the time of feeding. Nifedipine stocks were made fresh in Me₂SO; the final concentration of Me₂SO in the cultures was kept constant (0.5% v/v). Stock solutions of A23187 and ionomycin were made in ethanol; the final concentration of ethanol in the cultures was 1%. To determine the effects of low Ca²⁺, cells were grown in Chelex-treated serum (20) with Ca²⁺-free DMEM to which Ca²⁺ was added back as CaCl₂, or the cells were grown to confluence in DMEM:FCS, then transferred to serum-free keratinocyte growth medium (KGM, Clonetics, San Diego, CA) which was supplemented with CaCl₂. Ca²⁺ concentrations in the DMEM:FCS medium were measured by atomic absorption spectroscopy.

Analysis of Cell Extracts by SDS-PAGE and Western Blotting—Following experimental manipulation, the medium was removed and the cells harvested by scraping the dishes. Cells were homogenized (30 μl of buffer/cm² plate area) in 9 M urea, 50 mM Tris (pH 8.0), 10 μg/ml phenylmethylsulfonyl fluoride, 1 mM EDTA, and 0.1 mg/ml aprotinin (Sigma) in a ground glass homogenizer and centrifuged 4 min in a microcentrifuge (Beckman Instruments). Supernatants were removed, and 5-μl samples were analyzed by SDS-PAGE by the method of Laemmli (21), except that gradient gels from 4-15% acrylamide were used, or by two-dimensional O'Farrell gels with nonequilibrium electrophoresis in the first dimension as described by Hard and Scott (4). Because differentiation of keratinocytes involves degradation of cellular contents, as well as synthesis of large and variable amounts of keratins (1, 22), a constant proportion of each dish of cells was loaded into each lane of the gel. Gels were stained with Coo massie Brilliant Blue R-250 in 10% acetic acid and 50% isopropanol, destained in 10% acetic acid, and calibrated with low molecular mass standards (Pharmacia LKB Biotechnology Inc.) containing α-lactalbumin (14.4 kDa), pancreatic trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (68 kDa), and phosphorylase b (98 kDa).

For Western blotting, proteins resolved on gels were transferred to nitrocellulose (Schleicher & Schuell, Keene, NH) and probed with antibodies as described by Towbin et al. (23) with the following modifications. After transfer, the nitrocellulose was blocked with 2.5% powdered nonfat dried milk (Carnation, Los Angeles, CA), then reacted 1 h with primary antibody and 30 min each with goat antibody to rabbit IgG (Vector, Burlingame CA) and rabbit antibody to peroxidase (Vector) preincubated with excess peroxidase (Vector). The peroxidase was developed with H₂O₂ and 4-chloro-1-naphthol (Sigma) (24). Blots were probed with a rabbit antiserum raised against purified rat filaggrin which reacted with all filaggrin-related proteins (22) or with a rabbit antiserum raised against human calpain I (the generous gift of Dr. John Eke, Department of Biochemistry, Queen's University, Ontario, Canada). To determine if the broad filaggrin band represented population variation in size, reacted blots were cut into two strips which were boiled in Laemmli sample buffer with 9 μl urea added; extracted proteins were reanalyzed by SDS-PAGE and Western blotting. Western blots were calibrated with prestained standards.

RESULTS

Characterization of Processing in Cultured Keratinocytes—The rat keratinocyte cell line developed by Kubilus and Baden (19) expresses profilaggrin at confluence (22) and processes it as the cells stratify (26). Immunoprecipitation of radiolabeled proteins was precluded because of the insolubility of profilaggrin and the lability of profilaggrin and its products to proteases and phosphatases. To characterize the processing kinetics, cells were harvested at various times after confluence, solubilized in 9 M urea (which inhibits proteolysis and dephosphorylation), and analyzed by SDS-PAGE and Western blotting using a rabbit antiserum raised against rat filaggrin which reacts with profilaggrin, intermediates, and filaggrin (Fig. 1). Several bands were detected, including two which comigrated with rat epidermal profilaggrin and filaggrin. The size range of the filaggrin population varied from experiment to experiment, with the uppermost band corresponding to filaggrin from epidermis and the lower bands as much as 4000 Da smaller. In some cases individual bands can be discerned (see Figs. 1, 5, and 7), while in others the population variation is less dramatic (see Figs. 2 and 3). Below profilaggrin, smeared bands were detected which were shown to be similar in charge to profilaggrin on two-dimensional gels, suggesting they were the product of nonspecific proteolysis or turnover of profilaggrin.

Between profilaggrin and filaggrin, several bands were identified as true intermediates (labeled 2DI and 3DI in Fig. 1). The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; 2DI or 3DI, two- or three-domain intermediate; KGM, keratinocyte growth medium; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis.
by their basic pI as determined by two-dimensional gels (4). These intermediates were detectable on the 2nd day after confluence, while filaggrin was detectable on the 3rd day, supporting the identification of 2DI and 3DI as true intermediates and suggesting that processing occurs in two sequential stages, as previously observed in intact guinea pig and mouse epidermis (6, 12). A 50-kDa band was also observed (the band labeled * in Fig. 2), which is similar in size to an epidermal protein that reacted with antibody directed to the N-terminal Ca\(^{2+}\) binding domain of profilaggrin (18). Its first appearance coincided with filaggrin, but it reached a maximum before filaggrin did.

Culture of a large number of samples produced variability between the samples, apparently due to stacking of the dishes and to placement within the incubator. Processing was most reproducible when six-well dishes were placed close together on one rack. The most reliable comparisons were between samples in a single six-well plate, which limited the variables that could be compared. The half-maximum effective concentration of various effectors was estimated from initial experiments with a wide range of concentrations and repeated in a narrower range. The overall trends described below were reproducible, although the point of maximum sensitivity varied slightly between experiments.

Effect of Added Calcium—Because other events that occur during cornification are known to be regulated by Ca\(^{2+}\) (27), the influence of external Ca\(^{2+}\) on profilaggrin processing was examined. In DMEM:FCS, raising the [Ca\(^{2+}\)] increased the amount of filaggrin produced, with optimal processing at 10 mM Ca\(^{2+}\) (Fig. 2); titration of Ca\(^{2+}\) with EGTA inhibited second-stage processing (data not shown). Second-stage processing was sometimes partially inhibited by 20 mM Ca\(^{2+}\); the extent of that inhibition was variable. In addition, cells incubated in the higher levels of Ca\(^{2+}\) produced intermediates and filaggrin with apparent molecular masses on SDS-PAGE that were slightly larger than normal (marked with an asterisk (*) in Fig. 2).

The cell line did not produce significant amounts of profilaggrin when plated and grown to confluence in low Ca\(^{2+}\) conditions. To analyze the effects of Ca\(^{2+}\) levels below those in DMEM:FCS, cells were grown to confluence in normal DMEM:FCS (thus expressing profilaggrin), then transferred into Ca\(^{2+}\)-free DMEM with 10% Chelex-treated serum or into Ca\(^{2+}\)-free KGM. Calcium was added back at varying levels from 0.15 to 20 mM, and cell extracts were analyzed for profilaggrin expression and processing (Fig. 3). In 0.15–0.6 mM Ca\(^{2+}\), expression of profilaggrin was markedly decreased. To determine if the initiation of processing was the same at high and low Ca\(^{2+}\), the amount of sample analyzed at each time point was adjusted to approximately equal immunoreactivity. Intermediates appeared at the same time in high and low Ca\(^{2+}\), and the amount of the intermediates in a given sample corresponded to the amount of profilaggrin present in that sample (data not shown).

In cells grown in KGM with 1.2 mM Ca\(^{2+}\) (Fig. 3), expression of profilaggrin and processing to intermediates was similar to that seen in control cultures maintained in DMEM:FCS (Fig. 2). Over the range 1–20 mM [Ca\(^{2+}\)] in KGM, there was increased second-stage processing; no inhibition of the second-stage processing or altered molecular weight of intermediates and filaggrin was observed in cells cultured in KGM at 20 mM [Ca\(^{2+}\)], in contrast to what was seen in DMEM:FCS. At 20 mM Ca\(^{2+}\), processing was sometimes dramatically more complete in cells cultured in KGM (Fig. 3) than in serum-DMEM. Addition of the Ca\(^{2+}\) ionophores A23187 or ionomycin to cells in normal DMEM:FCS did not increase the amount of filaggrin detected (data not shown).

To explore the effects of Ca\(^{2+}\) in more detail, cells were transferred to a lower level (0.4 mM) of Ca\(^{2+}\) about 24 h after confluence. Profilaggrin expression was maintained and nor-

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**Fig. 2.** Induction of second-stage processing by Ca\(^{2+}\). Cells were grown to confluence in DMEM:FCS, then CaCl\(_2\) was increased to the concentration indicated at the bottom of the figure. Dishes were harvested 4 days later, and a constant proportion (2%) of each 35-mm dish was loaded in each lane of an SDS-PAGE. A, Coomassie-stained gel. B, Western blot probed with antibody to filaggrin. In higher Ca\(^{2+}\) levels, the intermediates and filaggrin have slightly larger apparent molecular masses (bands marked with *). fil indicates the position of rat filaggrin; 2DI and 3DI indicate the two- and three-domain intermediates; pro indicates profilaggrin; ? indicates the 50-kDa band (see legend to Fig. 1).

**Fig. 3.** Profilaggrin processing of cultured keratinocytes cultured in KGM at various [Ca\(^{2+}\)]. Cells were grown to confluence in DMEM:FCS, then transferred to Ca\(^{2+}\)-free KGM to which CaCl\(_2\) had been added. The final [Ca\(^{2+}\)] is indicated below each lane. After 4 days, cells were harvested and analyzed. Note the significantly greater processing to filaggrin in 20 mM Ca\(^{2+}\) compared to the results obtained in DMEM:FCS in Fig. 2. fil indicates the position of rat filaggrin; 2DI and 3DI indicate the two- and three-domain intermediates; pro indicates profilaggrin.
nal intermediates were produced, but there was very little second-stage processing to filaggrin (control in Fig. 4). One day later, when second-stage processing normally commenced in cells maintained in high Ca$^{2+}$, the [Ca$^{2+}$] was increased to 5 mM, and second-stage processing occurred. In fact, filaggrin levels were at least twice that normally seen. However, when Ca$^{2+}$ was added after 48 h in 0.4 M Ca$^{2+}$, no processing above background was induced (in the experiment shown, this level of Ca$^{2+}$ did produce a limited amount of second-stage processing in both the experimental and control samples). A similar loss of second-stage processing was observed when cells were repeatedly passaged (data not shown).

Effect of Calcium Channel Blockers and Protease Inhibitors—To determine whether calcium influx was responsible for the effects observed, the calcium channel blocker nifedipine (which selectively blocks Ca$^{2+}$ uptake via voltage-sensitive plasma membrane Ca$^{2+}$ channels) was tested for its effect on processing. Nifedipine blocked Ca$^{2+}$-induced filaggrin production in the presence of optimal levels of external Ca$^{2+}$ (Fig. 5), suggesting that Ca$^{2+}$ influx into the cytoplasm is involved in the activation of proteolysis. In some experiments, a lower yield of intermediates was also seen. However, interpretation of the effects of nifedipine is complicated by the known deleterious effects on cells. Cell death was assessed by the

![Fig. 4. Induction of processing by shifting confluent cells from low Ca$^{2+}$ to high Ca$^{2+}$. A, cells were grown to confluence in DMEM:FCS, then transferred to 0.4 mM Ca$^{2+}$ KGM. This concentration allowed expression of profilaggrin and normal production of intermediates. After 24 h in 0.4 mM Ca$^{2+}$ (48 h after confluence), CaCl$_2$ (open circles) or NaCl (closed circles) was added to a final concentration of 5 mM and cells were harvested at timed intervals (12 and 24 h) thereafter. B, as in A, except Ca$^{2+}$ was increased to 5 mM after 48 h in 0.4 mM Ca$^{2+}$ (72 h after confluence). + indicates cultures where no NaCl or CaCl$_2$ was added. In this experiment, some processing to filaggrin was observed at 72 h after confluence in 0.4 mM Ca$^{2+}$, but no additional processing was induced when more CaCl$_2$ was added. The times after the transfer into 0.4 mM Ca$^{2+}$ KGM are indicated below the lanes; the time of confluence was estimated, so that the designation of 24 h for the first sample is an approximation.

ability of cells to exclude trypan blue; the amount of cell death in nifedipine roughly correlated with the decrease in intermediates produced in nifedipine. In contrast, nifedipine did not prevent the alteration of molecular weight of filaggrin and intermediates seen in 20 mM Ca$^{2+}$ (Fig. 5), suggesting a separate mechanism was involved. A dose response study yielded half maximal inhibition of filaggrin production at 10 nM nifedipine, when added 12–24 h before processing began (Fig. 6). Because nifedipine is unstable, this represents an upper limit of the actual effective concentration of nifedipine that inhibited processing.

Further information on the nature of second-stage processing was obtained using the protease inhibitors pancreatic

![Fig. 5. Inhibition of second-stage processing by nifedipine. Cells grown in DMEM:FCS with 5 mM Ca$^{2+}$ and nifedipine at various concentrations were added 1 day after confluence, as described under "Materials and Methods." Cells were harvested on day 4 and analyzed. The filaggrin bands were quantified by densitometry of the Western blot. Values were corrected for slight differences in lane width. All values were within the linear range, as determined by scanning a second blot with known amounts of rat filaggrin in each lane.

![Fig. 6. Dose response of nifedipine on second-stage processing in cultured keratinocytes. Cells were grown in DMEM:FCS with 5 mM Ca$^{2+}$ and nifedipine at various concentrations was added 1 day after confluence, as described under "Materials and Methods." Cells were harvested on day 4 and analyzed. The filaggrin bands were quantified by densitometry of the Western blot. Values were corrected for slight differences in lane width. All values were within the linear range, as determined by scanning a second blot with known amounts of rat filaggrin in each lane.](image)
trypsin inhibitor, bestatin, pepstatin, chymostatin, and leupeptin. Only chymostatin and leupeptin were effective at inhibiting processing. Chymostatin inhibited both stages of processing, as had been previously observed and interpreted as inhibition of the first-stage endoproteinase (9). Leupeptin inhibited only second-stage processing. Treatment with leupeptin on the day of confluence both inhibited the generation of filaggrin and increased the accumulation of the intermediates (Fig. 7). This behavior is consistent with a model in which leupeptin inhibits only the second step of processing, leading to accumulation of the intermediates. In contrast, addition of leupeptin at day 3, after filaggrin was first detected, did not significantly inhibit second-stage processing (data not shown).

Altered Expression of Calpain I—Taken together, the results showing a Ca\(^{2+}\)-dependent cytoplasmic proteolysis and the sensitivity of that processing to leupeptin suggested that one of the Ca\(^{2+}\)-activated neutral proteases (calpains) may be involved in second-stage processing. To test whether calpain was activated in these cells, a polyclonal antibody raised against human calpain I was used to probe Western blots of cell extracts (Fig. 8). Preconfluent cells showed little immunoreactivity to human calpain I (data not shown). However, cells on the first day of confluence contained significant amounts of an immunoreactive 80-kDa band with mobility identical to that of the calpain I standard (the unactivated protease), as well as a slightly smaller amount of a 78-kDa band which has a molecular weight similar to that reported for activated calpain I (28). On the 4th day after confluence, when the cells were actively processing profilaggrin, the 80-kDa band had disappeared almost completely, leaving the 78-kDa band as the predominant component. Furthermore, at the time that calpain is maximally activated, several high molecular weight proteins can be seen to disappear on the gels (Fig. 2A); this observation is consistent with the manifestation of calpain activation in other cell types (29). These high molecular weight bands are also increased during leupeptin inhibition of second-stage processing (Fig. 7A). Treatment of the cells with nifedipine to inhibit second-stage processing of profilaggrin also blocked the proportional change in the calpain doublet, as well as the disappearance of the high molecular weight bands (not shown).

**DISCUSSION**

In this study, we have demonstrated that profilagrrin is processed in two independently regulated stages in a rat epidermal keratinocyte cell line. This is similar to processing seen previously in mouse (12) and guinea pig (6) epidermis, establishing this cell line as a useful model for probing the mechanism and regulation of processing events. The use of Western blots to detect the various profilaggrin products (which transfer with varying efficiency) and the lack of steady state in the system make it difficult to produce classical kinetic studies. Despite the limitations of the methodology, some conclusions can be made. First, the two stages are significantly separated in time, although the kinetics are different in the three systems (24 h in culture, 4–6 h in mouse epidermis, and 9–12 h in guinea pig epidermis). Second, only the second stage is acutely dependent on external Ca\(^{2+}\). Third, the two stages are pharmacologically distinguishable in that second-stage processing is selectively inhibited by leupeptin and nifedipine. Finally, under some culture conditions these cells lose the ability to undergo second-stage, but not first-stage, processing.

In the pharmacological studies, leupeptin produces the classical response expected for a specific inhibitor of the second-stage processing, in that inhibition of filaggrin production correlates with an increase in the intermediates, presumably because first-stage processing is occurring normally and the intermediates are accumulating. Nifedipine caused complete

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**Fig. 7.** Leupeptin inhibits second-stage processing with concomitant increases in intermediates. Cells were grown to confluence in DMEM:FCS and varying concentrations of leupeptin were added. Cells were harvested 4 days later. Lane 6, control; lanes 1, 2, 3, 4, and 5 contain 800, 160, 32, 6, and 1 μg/ml leupeptin, respectively. fil indicates filaggrin; 2DI and 3DI indicate intermediates; pro indicates profilaggrin. A, Coomassie-stained gel; B, Western blot.

**Fig. 8.** Complete conversion of calpain I to the activated form when second-stage processing is at a maximum. Cell extracts from confluent cultures were analyzed and Western blots probed with antibody to human calpain I (A) day 1 after confluence (no second-stage processing) or (B) 4 days after confluence (maximum second-stage processing). Extracts were analyzed on two-dimensional gels with nonequilibrium electrophoresis in the first dimension (right is acidic) and 4–12% SDS-PAGE in the second dimension. Prestained standards were included in the SDS-PAGE step in order to calibrate the gel and allow correct alignment of the calpain immunoreactive bands. Similar results were obtained with one-dimensional SDS-PAGE. On day 1, two bands are observed at 80 and 78 kDa, with 80 kDa being slightly stronger; on day 4 the 80-kDa band is almost undetectable. The calpain I standard, which represents the unactivated enzyme, comigrated with the 80-kDa band (data not shown). The only other reactivity was observed at the edge of the gel at the same molecular sizes, presumably from aggregated protein which did not enter the first gel.
inhibition of second-stage processing, with some decrease in first-stage processing apparently due to cell death. It might be argued that the decrease in the amount of intermediates is the cause of the inhibition of processing to filaggrin. However, the cellular concentration of intermediates remains the same after adjusting for cell death. Furthermore, filaggrin was produced in experiments where profilaggrin processing was limited and intermediates were present at a low yield (Figs. 1 and 3).

Second-stage processing was absolutely dependent on externally added calcium. Inhibition by nifedipine, which blocks L-type plasma membrane Ca\(^{2+}\)-channels in other tissues (reviewed in Ref. 30), implies that increased cytoplasmic Ca\(^{2+}\) induced by Ca\(^{2+}\) influx at plasma membrane channels is required for second-stage processing. The proposed role for a Ca\(^{2+}\) influx in the regulation of events of terminal differentiation is supported by the large increase in cytoplasmic Ca\(^{2+}\) that occurs in the transition zone of epidermis where the cells are cornifying (31). However, processing is not induced by calcium ionophores, suggesting that the increase in cytoplasmic Ca\(^{2+}\) must be gradual. Yusa and co-workers (32) have proposed that the rate of increase in cytoplasmic Ca\(^{2+}\) acts to coordinate several aspects of epidermal differentiation, including regulation of structural protein expression and formation of cornified envelopes. The hypothesis that there is an optimum [Ca\(^{2+}\)] for each phase of differentiation is supported by the experiments where keratinocytes were shifted from medium to high Ca\(^{2+}\) (Fig. 4). Shifting to high Ca\(^{2+}\) after first-stage processing has initiated (48 h) led to a higher level of filaggrin than when cells are shifted to high Ca\(^{2+}\) at confluence. Surprisingly, shifting at 72 h led to almost no filaggrin production. This window of responsiveness to Ca\(^{2+}\) suggests that the processing program is integrated with other events. Furthermore, cells are not continuously entering the differentiation pathway, but rather are progressing synchronously.

The inhibition of the Ca\(^{2+}\)-dependent second-stage processing by leupeptin implicates calpain (reviewed in Ref. 33) in second-stage processing. Calpain substrate specificity shows a preference for Leu, Ile or Val in the P2 site and a bulky residue in the P1 site (Met, Tyr, Arg, or Lys) at the carboxyl end of the scissile peptide bond (34, 35). There are potential calpain cleavage sites in the linker region. Preliminary studies of calpain I action on mouse profilaggrin yielded products consistent with cleavage in every linker region, rather than only the \(\alpha_2\) linker regions (not shown). This suggests that, although the first-stage proteolysis is specific for linker structure, the second-stage proteolysis is not; thus any linker segments uncleaved in the first stage will be cleaved in the second stage. Because the second-stage protease acts on intermediates, rather than profilaggrin, studies using keratin-intermediate complexes are required before concluding calpain I is the second-stage \(\alpha_2\) protease. Indeed, when leupeptin is added on day 3 after confluence (just as filaggrin first appears), second-stage processing is not inhibited. The lack of inhibition may be due to transport changes in the cornifying cell membrane that prevent uptake of leupeptin; alternatively, the \(\alpha_2\) protease may be a zymogen which is activated by calpain, but is not itself sensitive to leupeptin. More studies are necessary to clarify these events.

Calpain activation is associated with alterations in the cytoskeleton and with proteolysis of various cytoskeletal components in several cell types (see discussion in Ref. 29; examples include MAP 1 and 2, filamin and other actin binding proteins, and intermediate filament components). Immunohistochemical studies show an increased expression of calpain in the granular cells of epidermis (36), supporting the proposal that this protease plays a role in epidermal differentiation. A similar increase in calpain I expression is detectable in keratinocytes at confluence. When cultured rat keratinocytes begin second-stage processing, nearly all of the detectable calpain I is of the activated form. The lack of activation of second-stage processing in cells treated with calcium ionophores may reflect rapid activation and autodegradation of calpain I, rather than the slower turnover generated by a slow increase in cytoplasmic Ca\(^{2+}\) which has been proposed for normal differentiation (32).

It was surprising that processing of rat profilaggrin occurred in two stages, because structural analysis of rat profilaggrin showed that the primary structure of the linker segments of this profilaggrin are identical; however, the linker segments do differ in phosphorylation of residues located near the protease site (37). Variations in the phosphorylation may induce different three-dimensional structures of the linker segments. The three-dimensional features of the proteolytic site are as important as the specific cleavage sites in some proteases, for example, the substrates of several viral proteinases (38).

Two other observations relevant to processing were made in this study. First, in contrast to epidermis, the filaggrin population showed a size distribution of about 4000 Da, with the largest component similar in size to epidermally derived filaggrin. Analysis of the C and N termini of rat filaggrin showed that this protein was apparently subject to processing by exopeptidases (37). In culture there appears to be more extensive processing, possibly due to the longer time course of events. Second, abnormal processing occurred in 20 mM Ca\(^{2+}\)-DMEM:FCS. A likely explanation is that dephosphorylation is incomplete, which can markedly affect mobility on SDS-PAGE, and may also regulate first-stage processing in rat profilaggrin (37). Recently, increased cytoplasmic Ca\(^{2+}\) was shown to activate several growth factor-activated kinases (39). However, the effect on profilaggrin is only seen in DMEM:FCS, implying a serum component is involved, for example, thrombin, which is a known activator of growth factor activated-kinases (40).

The complex regulation of profilaggrin processing is likely to reflect a need for temporal order in the sequence of molecular events that characterize filament assembly. An extended meshwork or reticulate cytoskeleton is characteristic of transition cells, which may be the result of the interaction of intermediates with keratin filaments (4, 9). Processing of intermediates to filaggrin could trigger an orderly collapse of the extended network, generating the condensed cytoplasm of the mature cornified cell. The Ca\(^{2+}\)-dependent second-stage processing could coordinate the collapse with other Ca\(^{2+}\)-dependent events. These events include the formation of cornified envelope, which contains \(\epsilon-(\gamma\text{-glutamyl})\)lysine cross-links generated by a particulate Ca\(^{2+}\)-activated transglutaminase (reviewed in Ref. 41). Another major event in the transition zone is the exocytosis of the lamellar granules, whose contents form the lipid barrier between the cells (42). Although a specific role of Ca\(^{2+}\) in lamellar granule exocytosis has not been shown, it is interesting that calpain has been implicated in vesicle exocytosis in other systems (43), as well as in transglutaminase activation (44). Recently, it has been proposed that the Ca\(^{2+}\) binding domain of the N terminus of profilaggrin may play a role in coordinating the Ca\(^{2+}\)-dependent events of terminal differentiation (17, 18). If the 50-kDa band which is released early in second-stage processing (Figs. 1 and 2), does indeed contain the Ca\(^{2+}\) binding domain, its
release would be ideally timed to coordinate the Ca\textsuperscript{2+}-depend-
ent events.

Understanding the mechanisms by which calcium and ex-
ternal factors trigger profilaggrin processing in skin may
ultimately be important in defining the extracellular signals
which initiate terminal differentiation in keratinocytes as we11
as the coupling of the various events of cornification. As
knowledge of these profilaggrin-specific proteases accumu-
lates, their action and their regulation may also provide a
prototypical model system for the study of the role and control
of other specific cytoplasmic proteases.

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