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DIPEPTIDE INHIBITORS OF UBIQUITIN-MEDIATED PROTEIN TURNOVER PREVENT GROWTH FACTOR-INDUCED NEURITE OUTGROWTH IN RAT PHEOCHROMOCYTOMA PC12 CELLS

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Abstract. Dipeptide inhibitors of the ubiquitin-dependent proteolysis pathway governed by N-terminal recognition (N-end rule) in reticulocyte lysates significantly suppress NGF- and bFGF-induced neurite outgrowth in rat pheochromocytoma PC12 cells, but do not cause retraction of already formed neurites. Peptides which do not inhibit proteolysis are also without effect on PC12 cell differentiation. Suppression of neurite outgrowth is readily reversible upon removal of the inhibitors. These data demonstrate a requirement for specific protein turnover in the process of neuron-like differentiation in PC12 cells and provide the first demonstration of a physiological role for the N-end rule.

One of the major extralysosomal mechanisms for intracellular proteolysis in eukaryotic cells is the ubiquitin-mediated pathway (1-3). This process requires the formation of a multiubiquitin chain covalently linked to the ε-amino group of a lysine residue in the substrate protein (4). One way by which such proteins are recognized is through a destabilizing amino-terminal residue with an internal lysine residue in appropriate spatial juxtaposition to the amino terminus for the attachment of ubiquitin. Biochemical (5-7) and genetic (8-10) evidence indicates that this N-end rule-based identification (8, 11) is determined by sites on ubiquitin-protein ligase (E3) which are specific for bulky, hydrophobic N-terminal residues such as leucine, phenylalanine, tryptophan and tyrosine or for basic N-terminal residues such as histidine, arginine, and lysine. The absence of a significant phenotype in yeast *ubr1* mutants, which lack detectable E3 activity (10), as well as the known specificity of the co-translational N-terminal processing enzyme methionine aminopeptidase (11-17), which does not allow the formation of unstable N-termini directly, suggests that there may be only a limited number of natural substrates whose degradation occurs solely via the N-end rule pathway. In fact, although there is now good evidence from several

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Abbreviations used : NGF, nerve growth factor; bFGF, basic fibroblast growth factor.

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systems for ubiquitin-mediated proteolysis of a number of natural substrates (18-24), to date, only the Sindbis virus RNA polymerase has been shown to be targeted for proteolysis by the N-end rule recognition mechanism (25). Thus, the extent to which the N-end rule is actually utilized in *in vivo* situations is unclear.

PC12 cells, derived from a rat pheochromocytoma (26), have been widely used as a model system for studies of growth factor-induced neuronal differentiation. Treatment of PC12 cells with NGF or bFGF initiates a series of physiological events leading to their differentiation into sympathetic neuron-like cells characterized by neurite outgrowth (27). The biochemical pathways underlying this process, which are being intensively investigated in many laboratories (for reviews, see 28, 29), are initiated by receptor-bound tyrosine kinases which in turn activate a variety of other kinases (both serine/threonine and tyrosine-specific), phosphatases, lipases, etc. These signals are ultimately manifested in changes in gene expression and the phenotypic profile of the cell which lead to neurite outgrowth. The effects are both continuous and reversible, i.e. there is a constant requirement for factor to be present to maintain the differentiated state.

In this report, we show that dipeptide inhibitors, which specifically block the binding of N-end rule substrates to E3, prevent, but do not reverse, growth factor-induced differentiation suggesting that protein turnover is required for this process and providing the first definitive evidence for the participation of the N-end rule in a defined physiological event.

Materials and Methods

Chemicals - Amino acids and dipeptides were obtained from Sigma, Bachem Bioscience, Research Organics or Novabiochem. Bovine β-lactoglobulin and serum albumin (BSA) were from Sigma and United States Biochemical, respectively. Mouse NGF was prepared as β-NGF by the method of Bocchini and Angeletti (30); bFGF was a gift of the AMGEN Corp. Proteins were labeled with Na ^{125}I (ICN) as described previously (31). Rabbit reticulocytes were purchased from Green Hectares (Oregon, WI) and depleted of ATP and lysed as described by Hershko et al (32).

Assays of protein degradation - The breakdown of ^{125}I-labeled substrates was determined in crude reticulocyte lysates by measuring the production of radioactivity soluble in 5% (w/v) trichloroacetic acid (31). Dipeptides were added to a final concentration of 4 mM and bestatin [to inhibit dipeptide cleavage (5)] to a final concentration of 40 μg/ml.

Cell culture - Stock cultures of rat PC12 cells (originally obtained from D. Schubert, Salk Institute) were maintained in monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum and 5% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Neurite outgrowth assay - Cells were resuspended in complete DMEM at a density of 30,000 cells/ml and 2 ml were plated into each 35 mm well of 6-well clusters. The wells were precoated with rat tail collagen (33). After cell attachment (generally about 2 h), the medium was replaced with DMEM containing 1% horse serum to which various additions were made as specified. This medium was changed every 24 h. When present, NGF was added to a final concentration of 100 ng/ml and bFGF to 5 ng/ml. Dipeptides were added at various concentrations from stock solutions made in sterile water. Cells were scored as having neurites when processes extended two or more cell bodies in length from the periphery of the cell.

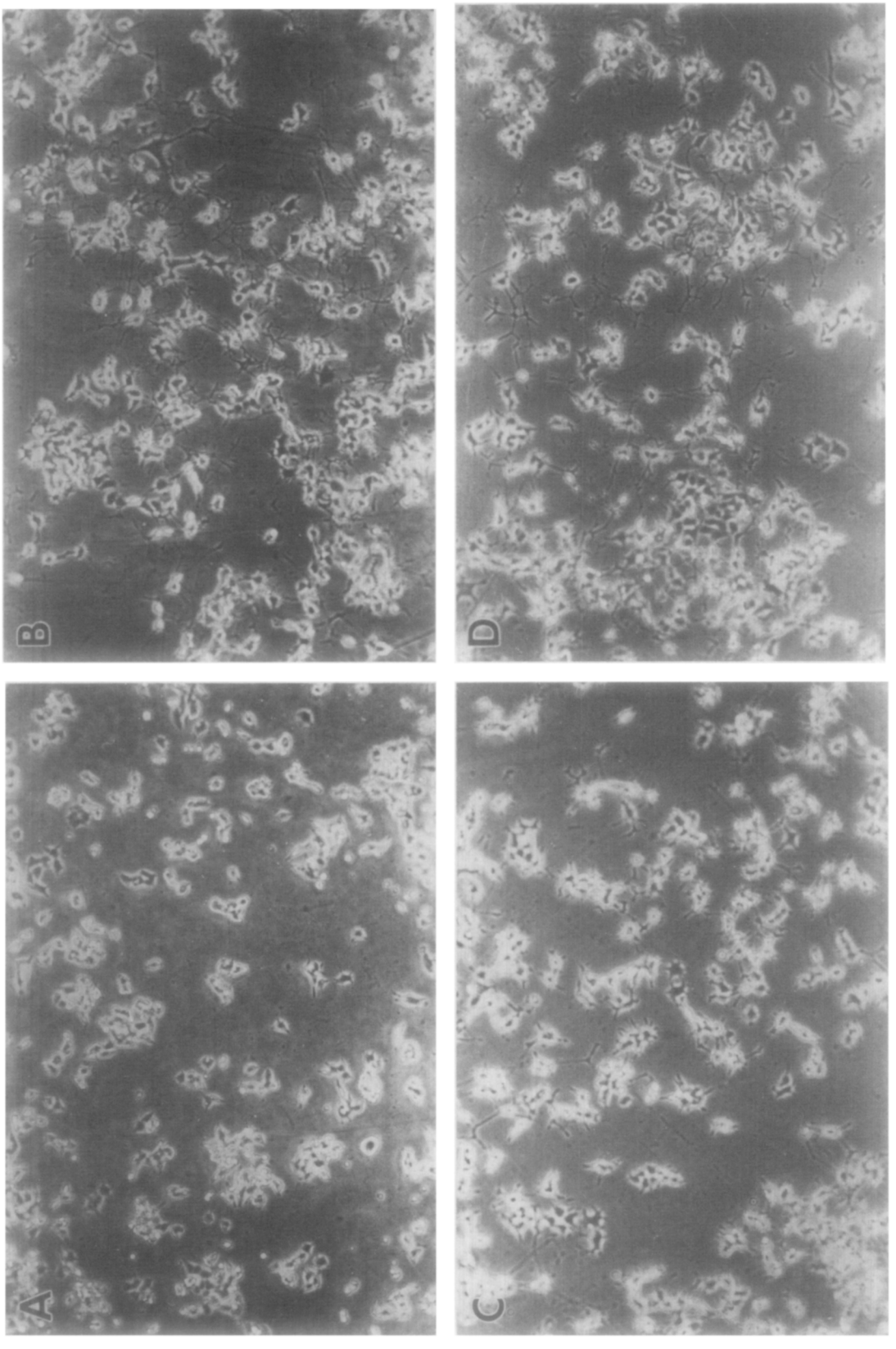
Results

The laboratories of Hershko (5, 7) and Varshavsky (6, 34) have shown that dipeptides with destabilizing N-terminal residues inhibit the binding of appropriate substrates to E3 and prevent

their degradation both in vitro and in vivo. Leu-Ala (all amino acids are the L-isomers, unless otherwise specified), for instance, inhibits the degradation of protein substrates containing bulky, hydrophobic N-terminal amino acids. Treatment of PC12 cells with NGF (100 ng/ml) (Figs. 1A and B) in the presence of Leu-Ala results in the suppression of neurite outgrowth by greater than 60% (Fig. 1C), whereas the addition of Ala-Leu at the same concentration does not affect NGF-induced neurodifferentiation (Fig. 1D) relative to controls. In cells stimulated with NGF in the presence of increasing concentrations of Leu-Ala, inhibition of neurite outgrowth was observed at dipeptide concentrations as low as 1 mM (Fig. 2). At the highest concentrations tested (40 mM), some cell death was observed between 48 and 72 h of treatment.

In order to further investigate the possible relationship between inhibition of NGF-induced neurite outgrowth and inhibition of N-end rule-directed proteolysis, we compared the effects of a number of dipeptides on the two pathways. ATP-dependent proteolysis was measured in reticulocyte extracts using ^{125}I-β-lactoglobulin, which has an N-terminal leucine as a model substrate containing a bulky, hydrophobic residue, and ^{125}I-BSA as a substrate for proteolysis mediated by the binding of a basic amino terminal residue to E3. BSA has an amino terminal aspartic acid, but it has been shown that acidic amino termini are secondary destabilizing residues and that proteins containing such termini are arginylated by arginyl-tRNA-protein transferase (6, 35) rendering them substrates for N-end rule-determined proteolysis via binding to the basic amino acid binding site of E3 (6, 36). Although subject to certain complications, such as the need for dipeptide uptake into PC12 cells and possible differences in the metabolic stability of the dipeptides in the two experimental systems, it is to be expected that only those dipeptides which inhibit proteolysis would also inhibit NGF-induced neurite outgrowth if the two pathways are linked. The data in Fig. 3 show that this is indeed the case. Leu-Gly, Leu-Ala, Leu-Val and Trp-Ala all inhibit the degradation of β-lactoglobulin and also inhibit neurite outgrowth. When the sequence is inverted, i.e. when leucine is the carboxyl-terminal amino acid of the dipeptide, neither proteolysis nor neurite outgrowth is inhibited. As shown previously by Reiss et al (5), when the N-terminal amino acid is of the D-configuration or is Nα-acetylated, the dipeptides do not inhibit proteolysis. D-Leu-Gly and N-acetyl-Leu-Gly both failed to inhibit the degradation of ^{125}I-β-lactoglobulin by reticulocyte extracts and NGF-induced neurite outgrowth (data not shown). Interestingly, Leu-Pro inhibits neither proteolysis nor neurite outgrowth. Previous studies which utilized dipeptides to probe N-end rule binding sites on E3 used dipeptides with a carboxyl-terminal alanine or glycine (5-7). These results indicate that the carboxyl terminal residue of the dipeptide and, by extension, the penultimate residue of a protein substrate, can influence binding to E3. Free leucine, tryptophan, glycine, alanine, valine and proline, alone or in combination and at the same concentrations, failed to inhibit either proteolysis or neurite outgrowth (data not shown).

Figure 1. Effect of Leu-Ala and Ala-Leu on NGF-induced neurite outgrowth in PC12 cells. Cells were incubated in DMEM plus 1% horse serum containing (A) no additions, (B) 100 ng/ml NGF, (C) 100 ng/ml NGF and 10 mM Leu-Ala, (D) 100 ng/ml NGF and 10 mM Ala-Leu. Medium was changed every 24 h and photographs were taken after 72 h.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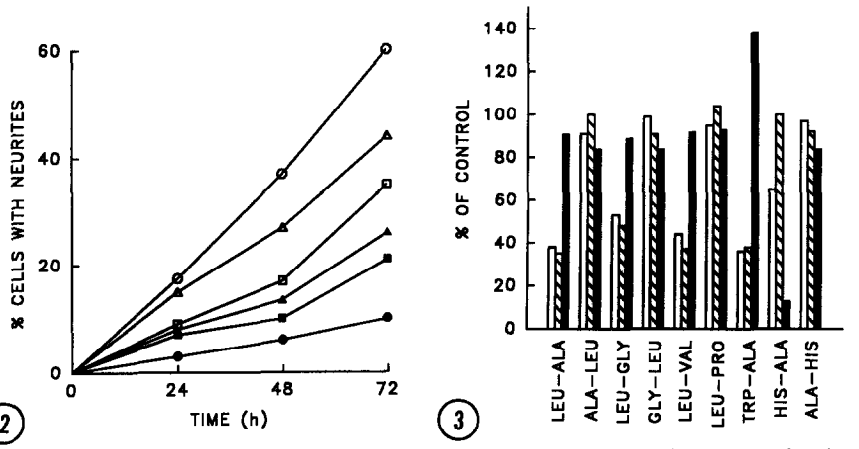

Figure 2. Inhibition of NGF-induced neurite outgrowth by Leu-Ala. PC12 cells were incubated in DMEM plus 1% horse serum and NGF (100 ng/ml) containing the following concentrations of Leu-Ala: 0 (○); 1 mM (△); 5 mM (□); 10 mM (▲); 20 mM (■); 40 mM (●). Cells were scored for neurites at the times indicated.

Figure 3. Effect of dipeptides on NGF-induced neurite outgrowth in PC12 cells and on degradation of ^{125}I-labeled proteins in reticulocyte extracts. The open bars represent the percentage of cells bearing neurites after 72 h of treatment with the indicated dipeptide compared to control cells treated with NGF only. 62-84% of these cells expressed neurites after 72 h. The effect of the dipeptides on degradation of ^{125}I-β-lactoglobulin and ^{125}I-BSA by reticulocyte extracts is represented by the hatched bars and solid bars, respectively. ATP-dependent degradation in the absence of dipeptides varied from 61-84%/h for ^{125}I-β-lactoglobulin and 20-33%/h for ^{125}I-BSA.

In agreement with earlier results (5), His-Ala (but not Ala-His) inhibited the degradation of ^{125}I-BSA by crude reticulocyte extracts. This dipeptide also inhibited NGF-induced neurite outgrowth, although not as strongly as the N-terminal leucine or tryptophan dipeptides (Fig. 3). Similar results were obtained with His-Gly (inhibitory) and Gly-His (not inhibitory) (data not shown). Free histidine, alone or in combination with free glycine and alanine did not inhibit either proteolysis or neurite outgrowth (data not shown). Gonda et al (6) have described a third E3 binding site in reticulocytes which recognizes proteins containing a serine, alanine or threonine at the N-terminal position. Since we did not find an effect of Ala-Leu or Ala-His, at concentrations as high as 20 mM, on NGF-induced neurite outgrowth, there may not be a role for proteolysis mediated by this E3 binding site in NGF-induced neurite outgrowth. However, Gonda et al (6) have noted that both Ala-Ala and Ala-Ser fail to inhibit degradation of appropriate substrates in reticulocyte extracts, so binding of dipeptides to this third E3 site may be too weak to inhibit neurite outgrowth.

A comparison of the growth rate of PC12 cells in DMEM plus 1% horse serum and in the same medium containing 100 ng/ml NGF or 100 ng/ml NGF plus 10 mM Leu-Ala showed no effect of either the growth factor or the dipeptide. In all three cases the cells grew with a doubling time of 52 h (data not shown), suggesting that inhibition of neurite outgrowth is not secondary to a more general effect on cell viability.

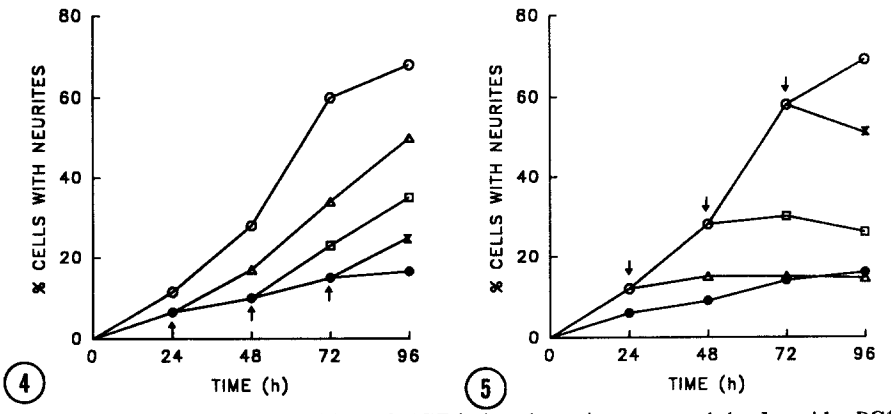

Figure 4. Reversibility of inhibition of NGF-induced neurite outgrowth by Leu-Ala. PC12 cells were incubated with NGF (100 ng/ml) and 10 mM Leu-Ala as described in the legends to Figures 1 and 2. (○) NGF; (●) NGF and Leu-Ala. At the times indicated (arrows), cells were washed twice with medium containing NGF but no Leu-Ala and incubation was continued in medium containing NGF alone. Leu-Ala removed at 24h (△); 48h (□); 72h (X)

Figure 5. Effect of Leu-Ala on neurite retraction in NGF-stimulated PC12 cells. 10 mM Leu-Ala was added to PC12 cells incubated in DMEM and 1% horse serum containing NGF (100 ng/ml) at the times indicated (arrows). Cells treated at O h (●); 24 h (△); 48 h (□) and 72 h (X). (○) control cells (no dipeptide added).

Dipeptide inhibition of neurite outgrowth is also readily reversible, further indicating that the inhibition of neurodifferentiation is not due to a toxic effect of Leu-Ala. Removal of Leu-Ala following up to 72 h of treatment allows rapid recovery of neurite outgrowth (Fig. 4). Furthermore, the dipeptides do not cause neurite retraction. As shown in Fig. 5, under conditions where cells were pretreated with NGF for various periods of time prior to treatment with dipeptides further neurite outgrowth was inhibited but there was no retraction of already formed structures.

The effects of bFGF on PC12 cells are very similar to those of NGF, although the signaling pathways triggered by these growth factors may differ in some respects (28, 29). It was, therefore, of interest to examine the effects of dipeptides on the bFGF-induced neurodifferentiation of PC12 cells. As shown in Fig. 6, bFGF-induced neurite outgrowth was also significantly inhibited by Leu-Ala but was not affected by Ala-Leu.

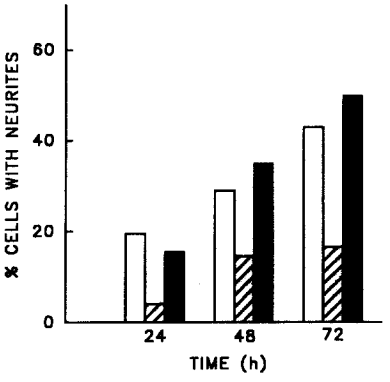

Figure 6. Effect of Leu-Ala and Ala-Leu on bFGF-induced neurite outgrowth. PC12 cells were incubated in DMEM plus 1% horse serum containing 5 ng/ml bFGF alone and 5 ng/ml bFGF plus 10 mM Leu-Ala or Ala-Leu (open, hatched or solid bars, respectively). Neurite outgrowth was scored at 24, 48 and 72 h.

Discussion

One well-established recognition signal for ubiquitin-mediated proteolysis is the N-end rule pathway (8, 11) in which proteins with destabilizing N-terminal amino acids are bound to E3, resulting in the formation of a multiubiquitin chain on an accessible lysine residue that targets the protein for degradation by the proteasome complex. The N-end rule pathway was elucidated by studies of the turnover of E. coli β-galactosidase and mouse dihydrofolate reductase (expressed as chimeric proteins that also contained a small N-terminal extension derived from an internal sequence of the lac repressor and ubiquitin) in yeast and reticulocyte lysates (6, 8, 9). Each construct was made so that the 20 different amino acids would occur at the N-terminus of the protein (after ubiquitin was removed in situ by ubiquitinase). To date, however, the only natural substrate determined to be degraded by the N-end rule pathway is the RNA polymerase of Sindbis virus (25). Although other proteins have been shown to be degraded in a ubiquitin-dependent manner, the signals which trigger their degradation remain unknown.

Reiss et al. (5) and Gonda et al. (6) have shown that the degradation of proteins potentially targeted for N-end rule directed proteolysis can be inhibited by dipeptides with an appropriate hydrophobic or basic residue in the amino terminal position, thus providing a possible means for assessing the involvement of E3 recognition (i.e. the N-end rule) in various physiological events. In whole cell paradigms, this requires the efficient uptake of the inhibitors. However, there is substantial evidence for both carrier-mediated facilitated transport and entry by simple diffusion of a wide variety of dipeptides in a number of mammalian cell types (37-39) suggesting access of exogeneously applied material to the appropriate intracellular environment should not be limiting. Indeed, the results presented support the view that dipeptide import and stability are not limiting in PC12 cells and that there is a complete correlation between peptides capable of inhibiting E3-mediated breakdown (through the hydrophobic site) and neurite outgrowth. Baker and Varshavsky (34) have carried out analogous experiments in yeast and have shown that dipeptides which inhibit proteolysis in reticulocyte extracts also inhibit the degradation of the appropriate E. coli β-galactosidase derivatives in intact cells.

Dipeptides specific for basic amino termini were also effective in blocking neurite formation but to a lesser degree than that seen with the hydrophobic agents. Since these two sites are independent, this result suggests that inhibiting the turnover of more than one protein can affect differentiation. Moreover, the weaker response to the basic dipeptides may reflect differences in import or stability rather than the importance of the substrate protein(s) to neurite formation. We have also tested several dipeptides not previously used in proteolysis inhibition studies. Of interest is the finding that Leu-Pro does not inhibit the degradation of ^{125}I-β-lactoglobulin, suggesting that the binding of inhibitors, and perhaps substrates as well, at the hydrophobic binding site of E3 is influenced by the adjacent amino acid residue. Leu-Pro also fails to inhibit NGF-induced neurodifferentiation of PC12 cells.

The reversible nature of the inhibition and the inability to cause retraction of already formed neurites indicates that the inhibitors are neither generally toxic nor do they act to block growth

factor signal transduction. If the latter were the case, the effect of the inhibitors would be equivalent to the removal of the growth factor stimulus which is well known to result in complete reversion to the undifferentiated phenotype (27). Therefore, they are more apt to affect the production of proteins required for neurite formation rather than differentiation itself. Possible candidates include proteins of the cytoskeleton or those required for substratum interaction. Since the effect presumably results from the build up of the affected protein(s), entities involved in regulation are equally likely to be involved. Determination of the germane substrates will provide the first identification of a cellular protein(s) whose half-life is controlled by the N-end rule pathway in situ.

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