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Short Contribution

Nerve growth factor induced neurite outgrowth from amphibian neuroepithelial precursor cells is prevented by dipeptides inhibiting ubiquitin-mediated proteolysis

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ABSTRACT The effect of dipeptides known to inhibit the ubiquitin-mediated proteolysis has been examined on growth factor induced neurite outgrowth from amphibian neuroepithelial precursor cells in primary culture. Nerve growth factor (NGF) stimulated neuritogenesis from these cells but fibroblast growth factor 2 (FGF-2) only increased the number of melanophores. The neurite outgrowth induced by NGF was inhibited by the dipeptides blocking the ubiquitin mediated proteolysis (Leu-Ala and Leu-Gly) whereas the inactive control dipeptides (Ala-Leu and Ala-His) had no effect. This suggests that ubiquitin-mediated proteolysis involving the ubiquitin ligase E3 is necessary for growth factor induced neuronal differentiation during the development of the central nervous system.

KEY WORDS: *neuroepithelial precursor cells, growth factors, neurite outgrowth, ubiquitin mediated proteolysis, ubiquitin ligase (E3)*

Ubiquitin-mediated degradation is an extralysosomal mechanism for protein turnover in eukaryotic cells (Hochstrasser, 1995). To be degraded through this pathway, a substrate protein is first modified by the covalent attachment of ubiquitin, a 76 residues protein that is widely distributed. The means for achieving this modification is the recognition of certain N-terminal sequences by ubiquitin ligase (E3), which facilitates the attachment of ubiquitin via specific ubiquitin transferases (E2) to produce polyubiquinitated structures. The actual proteolysis is carried out by the 26 S form of the proteasome. E3 recognizes bulky hydrophobic residues such as leucine or tryptophane and basic residues such as arginine or lysine, via independent sites. This process, named the N-End Rule (Varshavsky, 1992) has been well studied in reticulocyte lysates and in yeast but its precise physiological roles *in vivo* remain to be clearly elucidated. To date only the sindbis virus RNA polymerase (DeGroot *et al.*, 1991) and the G protein of yeast (Madura and Varshavsky, 1994) have been shown to be naturally degraded through this pathway. Dipeptides exhibiting hydrophobic or basic residues at the N terminal position can inhibit the binding of E3 to the substrate proteins competitively (Baker and Varshavsky, 1991). Thus Leu-Ala and His-Ala inhibit the degradation of lactalbumin and BSA, respectively, in a reticulocyte lysate. We have previously shown that dipeptides having an hydrophobic N terminal residue are able to specifically prevent growth factor-induced neurite outgrowth from rat pheochromocytoma PC12 cells (Hondermarck *et al.*, 1992). The target protein whose interaction with E3 is blocked by the inhibitory dipeptides is unknown. To determine whether this inhibition can be extended to neuronal cells during development, we have tested the activity of dipeptides that block binding to E3 on neurite outgrowth from amphibian neural plate derived cells in primary culture.

Growth factors effect on neurite outgrowth

As shown in Figure 1, nerve growth factor (NGF) stimulates neurite outgrowth from amphibian neuronal precursor cells. NGF stimulation of neurite outgrowth from neuroprecursor cells has already been reported (Mathieu *et al*, 1971). We show that the efficient dose is about 1 ng/ml (Fig. 2A) with a maximum effect at 10 ng/ml, which is substantially lower than what is commonly observed for other responsive cell types such as PC12 (Altin and Bradshaw, 1992). For a concentration of 10 ng/ml, a maximum effect was obtained after 5 days of stimulation (Fig. 2B). Instead of promoting neurite outgrowth, fibroblast growth factor 2 (FGF-2) was found to increase the number of melanophores (Fig. 1). No increase in the number of neurites was detected for any concentration of FGF-2 tested up to 100 ng/ml. FGF-2-induced

Abbreviations used in this paper: E3, ubiquitin ligase; FGF-2, fibroblast growth factor 2; NGF, nerve growth factor.

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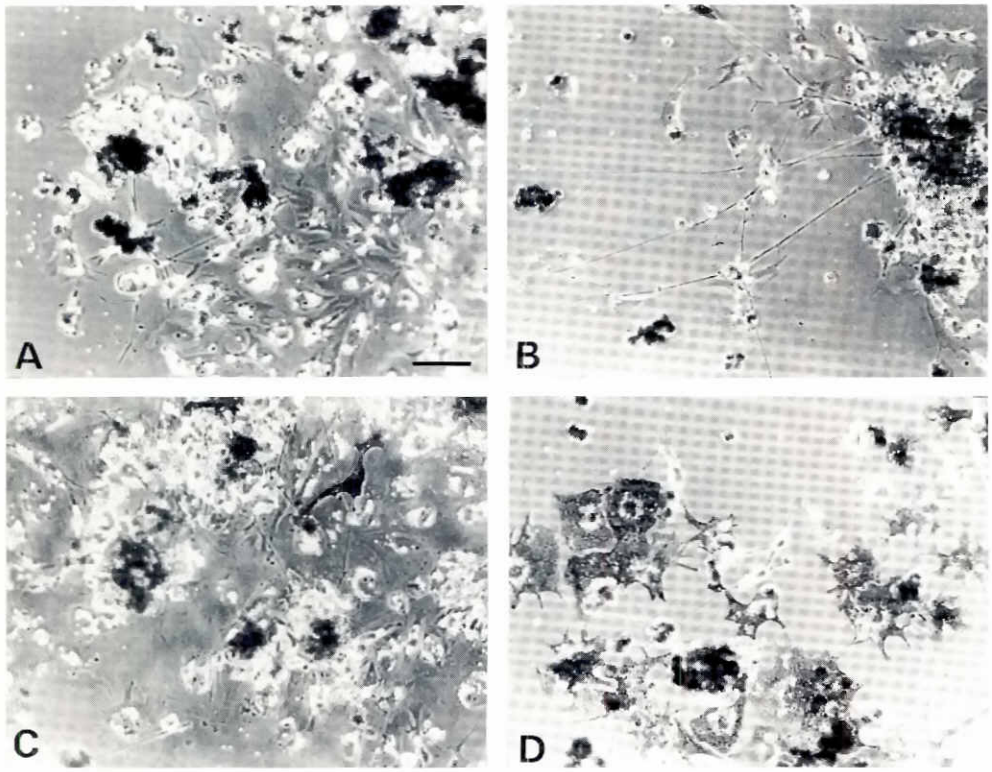

Fig. 1. Primary cultures from the amphibian neural plate and neural fold obtained as described in the text. (A) *Control.* **(B)** *NGF 10 ng/ml.* **(C)** *NGF Leu-Ala.* **(D)** *FGF-2 10 ng/ml. Pictures were taken after 5 days of treatment with growth factors and dipeptides under a phase contrast microscope. Bar, 20 μm.*

neurite outgrowth has been described for PC12 cells as well as on various neuronal cells during development (Sensenbrenner, 1993). However, FGF-2 stimulates cell proliferation without any neurite promoting activity on rat neuronal precursor cells (Gensburger *et al.*, 1987). In *Xenopus*, it has been clearly demonstrated that from stage 9+ to 10, FGF-2 stimulates neuritogenesis from ectodermal cells and then enhances melanophore production from stage 10 to $11^{1/2}$ (Kengaku and Okamoto, 1993). This is consistent with what we observed in the amphibian central nervous system since we found FGF-2 increasing only the number of melanophores in primary cultures derived from the early neurula stage. None of the other growth factors tested (epidermal growth factor, transforming growth factor ß and insulin-like growth factor I) showed any significant effect on either neuritogenesis or melanogenesis (data not shown).

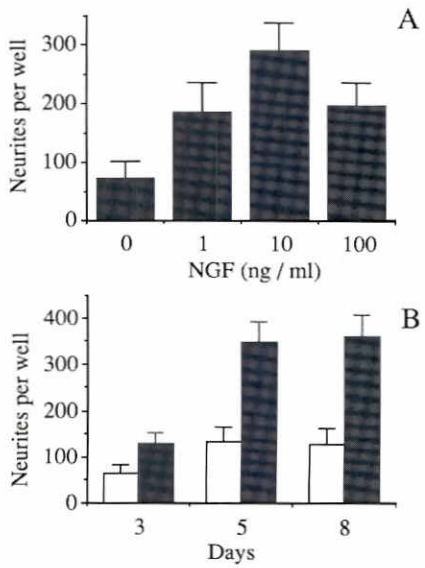

Fig. 2. NGF effect on neurite outgrowth (A) *Dose-effect of NGF on neurite number after 5 days of stimulation.* **(B)** *Effect of NGF at 10 ng/ml (black box) compared to the control situation (white box) after 3, 5 and 8 days of culture. Total number of neurites per 35 mm dish was determined under phase contrast microscope observation. Bars represent the mean±SD of 10 independent experiments. No NGF effect on the number of melanophores was found.*

Effect of dipeptides on NGF induced neurite outgrowth

The NGF-induced neurite outgrowth from these cells was inhibited by two dipeptides (Leu-Ala and Leu-Gly) known to inhibit

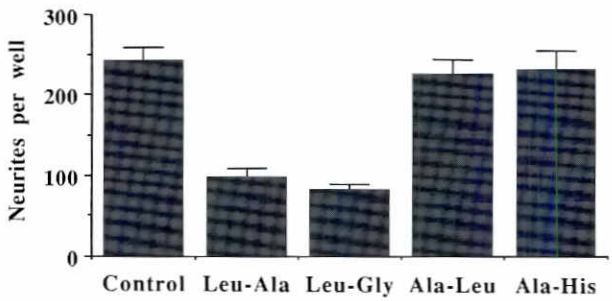

Fig. 3. Effect of dipeptides on NGF induced neurite outgrowth. *After 48 h of culture, NGF was added at the concentration of 10 ng/ml, with or without dipeptides, at the concentration of 20 mM. Neurites were counted in the whole 35 mm dishes after 72 h of treatment. Bars represent the mean±SD of 10 experiments.*

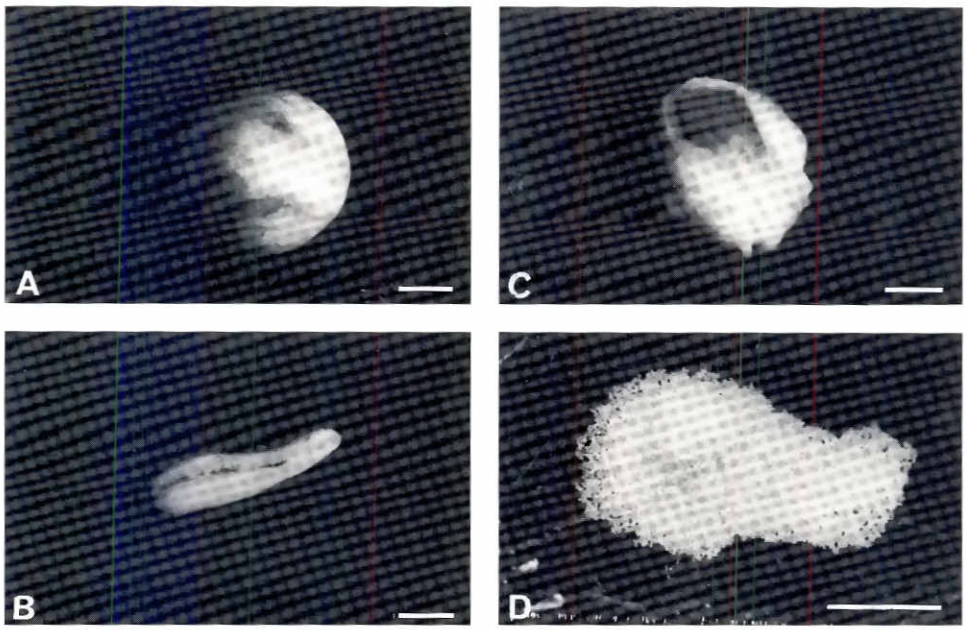

Fig. 4. Dissection of the *Pleurodeles* embryo. *Stage 14 eggs* **(A)** *were dissected to separate the neural plate and neural fold* **(B)** *from the rest of the embryo* **(C)**. *Neural plate and neural fold derived cells after incubation in the Steinberg dissociative buffer* **(D)**. *(See text for details). Bar, 0.6 mm.*

the binding between E3 and substrate proteins in the ubiquitin-mediated proteolysis process (Fig. 3). As a control situation, the dipeptides (Ala-Leu, Ala-His), which do not bind E3 as assayed in reticulocyte lysates, do not inhibit neurite outgrowth either. Changes in the amount of ubiquitin-protein conjugates are known to be concomitant with neuronal differentiation induced by NGF in PC12h cells (Takada *et al.*, 1994) and we previously demonstrated that these dipeptides (among others) prevent neurite outgrowth induced by NGF and FGF-2 in PC12 cells (Hondermarck *et al.*, 1992). The present study extends these latter observations to neuroprecursor cell differentiation and therefore demonstrates the involvement of E3 recognition in neurite outgrowth processes during development of the central nervous system. The molecular basis underlying this implication, i.e. the nature of the substrate proteins of E3, will now have to be studied.

Experimental Procedures

Primary cell cultures were derived from embryos (obtained by natural matings) of *Pleurodeles waltl* following the procedure described by Mathieu *et al.* (1971). Briefly, early neurulas (stage 14) were manually dissected to remove the jelly coat and vitelline membrane and the neuroectodermal cells from neural plate and neural fold were excised using platinum needles. After removal of the underlying chordomesodermal material, neuroepithelial cells were dissociated in Ca^{2+} and Mg^{2+}-free Steinberg solution (Tris HCl 50 mM, pH 8.7; NaCl 58 mM, KCl 0.6 mM, EDTA 0.9 mM). After complete dissociation, cells were transferred to 35 mm collagen coated dishes containing 70% Leibovitz medium (L15 Sigma) supplemented by 5% horse serum and 2% of penicillin-streptomycin. Photographs taken at the different steps of the dissection are shown in Figure 4. Two embryos were used for each 35 mm dish. After 48 h, the cells were attached, spread out over the collagen substratum, and growth factors and/or dipeptides were added at the indicated concentration. NGF was prepared as 2.5 S NGF from mouse submaxillary glands by the method of Bocchini and Angeletti (1969). FGF-2 was a gift from Amgen Corp. (Thousand Oaks, CA, USA). Dipeptides were from Research Organics Inc. (USA). Cultures were kept at 20°C and new medium, with or without growth factors and dipeptides, was supplied every two days. Neurites and melanophores were counted under the microscope. For each dish, all neurites were counted. Prolongations with a length corresponding to at least 2-fold the size of cell body were considered as neurite.

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