Osteogenic Protein-1 Regulates L1 and Neural Cell Adhesion Molecule Gene Expression in Neural Cells*

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Osteogenic protein-1 (OP-1) is a member of the TGF-β superfamily that is expressed in the nervous system. We recently showed that human recombinant osteogenic protein-1 (hOP-1) strongly promotes the aggregation of dividing neuroblastoma x glioma hybrid NG108-15 cells, in part by inducing the major isoforms of the neural cell adhesion molecule (N-CAM) (Perides, G., Safran, R. M., Rueger, D. C., and Charness, M. E. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 10326–10330). Here, we show that hOP-1 induces N-CAM gene expression approximately 6-fold in NG108-15 cells without changing the levels of N-cadherin, neurofilament 200, Thy-1, tau, and Goα. OP-1 induction of L1 and N-CAM was unassociated with changes in cell proliferation and was not reproduced by cellular differentiation. The increased adhesiveness of hOP-1-treated NG108-15 cells could be inhibited in part by Fab fragments of an anti-L1 polyclonal antiserum. L1 and N-CAM expression first increased 12–18 h after hOP-1 treatment, reached a maximum after 2–3 days, persisted for up to 5 days, and returned to control levels 3 days after hOP-1 withdrawal. The increases in L1 and N-CAM protein levels were preceded or accompanied by large increases in the abundance of L1 and N-CAM mRNAs. Actinomycin D prevented the induction by hOP-1 of L1 and N-CAM mRNAs, suggesting that hOP-1 regulates immunoglobulin CAM gene transcription. OP-1 is the first described growth factor that regulates both N-CAM and L1 gene expression.

N-CAM and L1 are calcium-independent immunoglobulin cell adhesion molecules (IgCAMs) that have been implicated in neurulation, neurite outgrowth, neuronal migration, fasci- culation, synaptogenesis, myelination, and neural regeneration (1). N-CAM is expressed early in development in dividing neuroblastoma x glioma hybrid NG108-15 cells, in part by inducing the major isoforms of the neural cell adhesion molecule (N-CAM) (Perides, G., Safran, R. M., Rueger, D. C., and Charness, M. E. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 10326–10330). Here, we show that hOP-1 induces N-CAM gene expression approximately 6-fold in NG108-15 cells without changing the levels of N-cadherin, neurofilament 200, Thy-1, tau, and Goα. OP-1 induction of L1 and N-CAM was unassociated with changes in cell proliferation and was not reproduced by cellular differentiation. The increased adhesiveness of hOP-1-treated NG108-15 cells could be inhibited in part by Fab fragments of an anti-L1 polyclonal antiserum. L1 and N-CAM expression first increased 12–18 h after hOP-1 treatment, reached a maximum after 2–3 days, persisted for up to 5 days, and returned to control levels 3 days after hOP-1 withdrawal. The increases in L1 and N-CAM protein levels were preceded or accompanied by large increases in the abundance of L1 and N-CAM mRNAs. Actinomycin D prevented the induction by hOP-1 of L1 and N-CAM mRNAs, suggesting that hOP-1 regulates immunoglobulin CAM gene transcription. OP-1 is the first described growth factor that regulates both N-CAM and L1 gene expression.

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¶ The abbreviations used are: CAM, cell adhesion molecule; IgCAM, immunoglobulin cell adhesion molecule; MeSO, dimethyl sulfoxide; OP, osteogenic protein; hOP-1, recombinant human osteogenic protein-1 precursor protein; BMP, bone morphogenetic protein; kb, kilobase(s); PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinethanesulfonic acid; mAb, monoclonal antibody.

The decapentaplegic-Vg1-related subfamily of the TGF-β superfamily comprises a structurally related group of morphogens, and one of these, Drosophila decapentaplegic, regulates homeobox gene expression (26). The observation that osteogenic protein-1 (OP-1), a member of the decapentaplegic-Vg1-
related subfamily, is expressed in the nervous system (27-29) and led us to examine whether it would regulate N-CAM levels in neurons. NG108-15 recombinant human OP-1 (hOP-1) increased by 6- to 30-fold the expression of all three major isoforms of N-CAM in NG108-15 neuroblastoma × glioma hybrid cells. These changes were associated with an increase in cell adhesion and the formation of proliferating cells of epithelioid sheets and multilayered cell aggregates (30). The failure of anti-N-CAM antibodies to fully inhibit either the morphoregulatory properties of hOP-1 or the calcium-independent adhesion of hOP-1-treated cells (30) raised the possibility that hOP-1 induces additional calcium-independent CAMs.

In this report we show that hOP-1 strongly and selectively induces the expression of L1 and N-CAM in NG108-15 cells by increasing the abundance of L1 and N-CAM mRNA. hOP-1 appears to act by a transcriptional mechanism of gene regulation that is independent of cell differentiation.

**EXPERIMENTAL PROCEDURES**

**Materials—**Antibodies were purchased from the following sources: rat monoclonal antibody H28.123 against N-CAM (31) from AMAC Inc. (Westbrook, ME); mAb against the 200-kDa neurofilament protein from Boehringer Mannheim; mAb against Thy-1 from Chemicon (Temecula, CA); rh-giant rat anti-rat antibody from ICN (Irvine, CA); and rabbit antisera RMI against Go, protein from DuPont NEN. Generous gifts of antibodies were provided as follows: mAb 74–SH7 against L1 (32) from Dr. V. Lemmon (Case Western, Cleveland, OH); Fab fragments of a rabbit polyclonal antibody against L1 (33) from Dr. F. Rathen (Universität Hamburg, Germany); two antibodies against N-Cadherin from Dr. J. Lifshitz (Clemson University, SC) and from Dr. M. Takeichi (Kyoto, Japan). Goat anti-mouse and goat anti-rabbit antibodies conjugated with horseradish peroxidase, fluorescein, or rhodamine were obtained from Cappel Organon Teknica (Durham, NC). hOP-1 and human recombinant OP-1 precursor protein (opoP-1) were isolated from conditioned medium from transfected Chinese hamster ovary cells by anion exchange chromatography followed by reverse phase high performance liquid chromatography to greater than 98% purity, as determined by SDS-PAGE (36). All other chemicals were from Sigma and were of analytical grade.

**Cell Adhesion Assay—**NG108-15 cells were cultured for 3 days on poly-D-lysine-coated 6-well trays (Corning, NY) containing chemically defined medium containing 0 or 40 ng/ml of hOP-1. Cells were labelled with acetoxy at -20 °C for 20 min and stored at -20 °C until used. 30,000 cells were incubated for 30 min with TBS containing 10 mM l-tirosine and 0.1% bovine serum albumin followed by 1 h with mAb H28.123, washed 3 times for 5 min with TBS, and incubated for 1 h with a goat anti-rat fluorescein-conjugated antibody. Control and hOP-1-treated cells were photographed at 100 × magnification using identical exposure times.

**Western Blot Analysis—**NG108-15 cells were cultured for 3 days in defined medium containing 0-100 ng/ml of hOP-1. Cells were harvested in TBS, and protein was determined by the method of Bradford (38). Cells were solubilized with 5 × concentrated SDS-sample buffer (30), and 60-120 μg of protein from each well were separated by 5-15% SDS-PAGE (39) and transferred electrothermically to nitrocellulose (40). The nitrocellulose membranes were blocked with TBS containing 0.1% bovine serum albumin and 0.1% Tween 20 (blocking buffer), incubated for 2 h at room temperature with the primary antibody (against N-CAM, L1, THY-1, neurofilament protein 200 kDa, tau, or Go), washed 3 times for 5 min with blocking buffer, and then incubated for 1 h at room temperature in blocking buffer containing 0.05 μCi/ml 125I-goat anti-rat IgG (N-CAM) or horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies. The membranes were then washed three times for 5 min with blocking buffer and subjected to autoradiography (N-CAM), stained with 3'-3' dianisobenzine (L1), or incubated with chemiluminesence reagents prior to film exposure (C).

**Immunofluorescence—**Total cellular RNA was isolated from NG108-15 cells by the RNAzol B method (Biotex Lab., Inc.; Houston, TX) (41). RNA was dissolved in diethyl pyrocarbonate-treated water, its concentration was measured by absorbance at 260 nm, and it was stored at -70 °C until further use. Ten to 20 μg of RNA was denatured in 10% formaldehyde, 50% formamide, 20 mM MOPS, pH 7.0, and fractionated by electrophoresis on 1.2% agarose gels containing 0.3% formaldehyde, 20 mM MOPS, pH 7.0. Gels were stained for 15 min with 33 μl/ml acridine orange in phosphate-buffered saline, pH 6.5, and photographed to verify the integrity and amount of RNA. The RNA was transferred to nylon membranes (Bio-Rad ZetaBond GT) by capillary blotting and cross-linked by baking for 1 h at 80 °C. Membranes were prehybridized for 2 h at 42 °C with 50% formamide, 20 μg/ml salmon sperm DNA, 2.5 × Denhardt's solution (1 × Denhardt's = 50 mg/ml Ficol, polynucleoprotein, and bovine serum albumin), 0.5% SDS, and 0.1 × SSPE (1 × SSPE = 150 mM NaCl, 10 mM NaH2PO4, pH 7.4, 1 mM EDTA) and then hybridized overnight at 42 °C with 1-5 × 106 cpm/ml of the human N-CAM and L1 32P-labeled cDNA probes. Probes for hybridization were radiolabeled by hexamer labeling (42) and purified on Select D (5'-3' Inc.; Boulder, CO). Following hybridization, membranes were washed successively at 65°C in 0.1 × SSPE, 20 mM NaCl, 15 mM sodium citrate, pH 7.0, 1 × and 0.5 × SSC, each containing 0.2% SDS, and exposed to radiographic film (Kodak X-Omat AR) with two intensifying screens for 1-14 days at -70 °C. Autoradiograms were analyzed by scanning densitometry. Filters were rehybridized with probes from unselected and 20% 74H7 mouse mAb against L1, washed 3 times for 5 min with TBS, and incubated for 1 h with a goat anti-mouse rhodamine-conjugated antibody. Control and hOP-1-treated cells were photographed at 100 × magnification using identical exposure times.
Effect of hOP-1 on Cell Proliferation and Neurite Extension—Members of the TGFβ superfamily have significant effects on dividing cells, promoting or inhibiting proliferation in different tissues (43). To determine whether hOP-1 affects cell proliferation, logarithmically dividing NG108-15 cells were cultured for 1–5 days with 0 or 40 ng/ml hOP-1, harvested, and counted in a hemocytometer. The mean doubling time of log-phase control and hOP-1-treated cells was 23.0 ± 1.9 and 22.9 ± 0.2 h, respectively (Fig. 1A). The protein content/well of cells treated with 40 ng/ml hOP-1 was 94 ± 5% (n = 12) of that of control cells. The viability of control and hOP-1-treated cells was greater than 95%. Although many cells treated with hOP-1 exhibited a lengthening of small processes without growth cones, only a small percentage of cells extended neurites with growth cones (Fig. 1B). In general, the percentage of neurite-bearing cells increased as a function of the concentration of hOP-1. However, in some experiments, high concentrations of hOP-1 caused most cells to associate in tightly adhering, multicellular clusters, from which few neurites extended. The induction of neurite extension by hOP-1 was less than that achieved by forskolin or MeS0, which, in contrast to hOP-1, also reduced cell proliferation. These data indicate that hOP-1 treatment does not reduce the proliferation, viability, or protein content of NG108-15 cells and, for the majority of cells, does not induce differentiation.

Increase in L1 Expression—To determine the effects of hOP-1 on L1 expression, NG108-15 cells were cultured for 3 days in the absence and presence of 40 ng/ml hOP-1 and processed for L1 and N-CAM immunofluorescence. Control NG108-15 cells exhibited low levels of L1 and N-CAM immunoreactivity (Fig. 2, a and b) that increased greatly after treatment with hOP-1 (Fig. 2, c and d). Double immunofluorescence staining of hOP-1-treated cells demonstrated a predominance of immunoreactivity for both IgCAMs in areas of cell-cell contact. Prominent L1 immunostaining was also present along the course of neurites.

The induction of L1 by hOP-1 was verified by Western blot analysis. NG108-15 cells exhibited low constitutive expression of the 200-kDa band identified with anti-L1 mAb 74-5H7. Treatment of NG108-15 cells for 3 days with varying concentrations of hOP-1 produced a large, concentration-dependent increase in the density of the 200-kDa band. This effect was relatively specific; Western blots using antibodies against neurofilament 200, tau, thy-1, and Gαo (the α subunit of the G protein that stimulates adenylyl cyclase) did not disclose any induction of these proteins by hOP-1 (Fig. 2f). Using two different antibodies, we were unable to detect any induction of N-cadherin (not shown).

Inhibition of Cell Aggregation with L1 Antibodies—To evaluate whether L1 contributes to the increased adhesiveness of hOP-1-treated cells, we performed short-term aggregation assays using single cell suspensions of NG108-15 cells that had been cultured for 2 days in the absence and presence of 10 ng/ml hOP-1. The cells were harvested, dispersed in a calcium-free buffer, rotated at 40 rpm for 30 min at room temperature, and then observed under phase contrast microscopy. From a single cell suspension, 14 ± 1% (n = 3) of control cells and 76 ± 8% of hOP-1-treated cells reassociated in clusters of two or more cells. In contrast, when hOP-1-treated cells were preincubated for 30 min on ice with Fab fragments of an anti-L1 polyclonal antibody, aggregates formed in only 39 ± 9% of cells (Fig. 3). The inhibition of cell adhesion by this anti-L1 antibody was comparable with what we observed previously for an anti-N-CAM antibody (30), indicating that L1 is an important mediator of the cell-cell interactions of hOP-1-treated NG108-15 cells.

Effect of Differentiation and Phorbol Esters on IgCAM Expression—To explore the relationship between differentiation and IgCAM expression, we treated NG108-15 cells with 1 μM forskolin or 1% MeS0 to induce differentiation (44). Both forskolin and MeS0 decreased cell proliferation (not shown) and caused neurite extension (Figs. 1B and 4). The percentage of cells extending neurites was greater after treatment with forskolin or MeS0 than with hOP-1; however, only hOP-1-treated cells showed increased expression of N-CAM and L1 (Fig. 5A). hOP-1 induced N-CAM and L1 to the same extent in dividing and in differentiated cells (Fig. 5A), providing additional evidence that this action of hOP-1 is independent of the cell cycle.

Forskolin increases cAMP levels in NG108-15 cells by activating adenylyl cyclase. The failure of forskolin to increase the expression of N-CAM or L1 suggests that hOP-1 does not regulate IgCAM expression by activating protein kinase A.

We next asked whether protein kinase C participates in this process. NG108-15 cells were treated for 1, 24, or 48 h with 10 nM phorbol 12-myristate 13-acetate, a phorbol ester activator of protein kinase C, and were evaluated after a total of 48 h. None of these treatments changed cell morphology (not
**FIG. 2.** Effect of hOP-1 on the expression of L1, N-CAM, and other proteins. NG108-15 cells were cultured for 3 days on poly-D-lysine-coated glass coverslips in the absence (a and b) or presence (c and d) of 40 ng/ml hOP-1. The cells were fixed in acetone at \(-20^\circ C\) and stained with the rat mAb H28.123 against N-CAM (a and c) and the mouse mAb 74-5H7 against L1 (b and d). Photographs were taken with identical exposures. Note the low level of staining shown or increased the expression of N-CAM or L1 (Fig. 5A). These data imply that N-CAM and L1 are not regulated in NG108-15 cells by either protein kinases A or C and suggest that hOP-1 produces its effects by an alternative signaling pathway.

**Induction of IgCAMs by the OP-1 Precursor Protein**—Members of the TGF-\(\beta\) superfamily are translated as inactive precursor homodimers that are then cleaved by specific proteases to produce biologically active, disulfide-linked homodimers. The primary translation product of OP-1 mRNA is a protein of 431 amino acids, including a putative signal peptidase site at amino acid 29 and a maturation site at position 292 (27). To compare the biological activity of the OP-1 precursor protein (pOP-1; \(M_r \approx 116\) kDa) and the mature recombinant protein (hOP-1; \(M_r \approx 36\) kDa), we cultured NG108-15 cells for 3 days in the presence of 1.2 nM of recombinant pOP-1 or hOP-1 (120 and 40 ng/ml, respectively). Interestingly, pOP-1 exhibited the same morphoregulatory activity as hOP-1 (Fig. 4). Treatment of NG108-15 cells with 1.2 nM pOP-1 caused 58 ± 8% of the cells to associate in clusters of three or more tightly adherent cells, as compared with 52 ± 3% of hOP-1-treated cells. Moreover, pOP-1 induced N-CAM and L1 to the same extent as hOP-1 (Fig. 5). These data imply that the precursor protein is either cleaved by NG108-15 cells or, in contrast to other members of the TGF-\(\beta\) superfamily, has the same biological activity as the mature protein.

**Kinetics and Mechanism of L1 and N-CAM Induction**—We next studied the kinetics of IgCAM induction by hOP-1 and asked whether there were associated changes in IgCAM mRNA. NG108-15 cells were cultured for 30 min to 4 days with 40 ng/ml of hOP-1. Cells were solubilized in SDS sample buffer and subjected to Western analysis, and total RNA was isolated from parallel cultures for Northern analysis. Expression of N-CAM and L1 increased rapidly between 12 and 24 h after treatment with hOP-1 and reached a maximum after 2–3 days (Fig. 6). Hybridization of total RNA from control NG108-15 cells revealed faint bands of approximately 6.7 and 7.2 kb, in keeping with the low constitutive expression of N-CAM-140 and N-CAM-180, respectively (30). Levels of the 6.7-kb transcript exceeded those of the 7.2-kb transcript, consistent with the greater abundance in NG108-15 cells of N-CAM-140 compared with N-CAM-180. Treatment of NG108-15 cells with hOP-1 caused a striking increase in the abundance of all detectable N-CAM transcripts. This effect.
FIG. 4. Effect of MeSO₂, forskolin, hOP-1, and precursor hOP-1 on the morphology of NG108-15 cells. NG108-15 cells were cultured for 3 days in defined medium in the absence (a) and presence (b) of 1% MeSO₂, 1 µM forskolin (c), 40 ng/ml (1.2 nM) hOP-1 (d), or 120 ng/ml (1.2 µM) recombinant OP-1 precursor protein (e). Note the long neurites in cells treated with MeSO₂ and forskolin, hOP-1- and pOP-1-treated cells show the same degree of aggregation.

FIG. 5. Effect of MeSO₂ (DMSO), forskolin, phorbol 12-myristate 13-acetate, hOP-1, and OP-1 precursor protein on IgCAM induction. A, NG108-15 cells were cultured for 3 days in the absence (control), and presence of 40 ng/ml hOP-1 (OP-1), 1% MeSO₂, or 1 µM forskolin. After 3 days, the cells were harvested and solubilized with SDS-sample buffer, and 80 µg of protein were separated by 5-15% SDS-PAGE, transferred to nitrocellulose, and immunostained with the 74-5H7 mAb against L1. Note that MeSO₂ and forskolin have no effect on N-CAM and L1 expression. Cells cultured in the absence or presence of 10 nM phorbol 12-myristate 13-acetate, 40 ng/ml hOP-1, or 120 ng/ml pOP-1 were harvested after 2 days and subjected to immunoblot analysis with mAbs H28.123 or 74-5H7. 1 µM forskolin increased cAMP levels by 49-fold, whereas MeSO₂ did not change cAMP levels (not shown). The experiments were repeated three times with similar results. B, concentration-dependent induction of L1 by hOP-1 and pOP-1. NG108-15 cells were incubated for 3 days with the indicated concentrations of hOP-1 or recombinant pOP-1. Cells were solubilized, and 80 µg of protein were fractionated by 5-15% SDS-PAGE, transferred to nitrocellulose, and immunostained with the 74-5H7 mAb against L1. Shown is the densitometric analysis from a representative experiment that was repeated three times with similar results.

was observed as early as 30 min after treatment with hOP-1 and reached a maximum after approximately 48 h. The proportions of the different N-CAM transcripts were similar in control and hOP-1-treated cells.

Hybridization with the L1 cDNA revealed low expression of a single transcript of approximately 6 kb (Fig. 6), consistent with the reported size of L1 mRNA (6). hOP-1 caused a large increase in the abundance of L1 mRNA, beginning after 30 min and reaching a peak after 48 h. As was the case for N-CAM, the increase in L1 mRNA preceded the increase in L1 protein, suggesting that changes in the abundance of the IgCAM mRNAs contributed to the increases in the encoded proteins.

To investigate whether the effects of hOP-1 are reversible, NG108-15 cells were incubated for 3 days with 40 ng/ml hOP-1 and harvested 1, 2, and 3 days after the removal of hOP-1 for Western and Northern analyses. High levels of N-CAM and L1 mRNA persisted for 1 day after the removal of hOP-1 and declined rapidly after 2 days (Fig. 7a). Similarly, N-CAM and L1 protein levels remained elevated for 24 h and returned gradually to control levels after 3 days (Fig. 7b). Interestingly, the removal of hOP-1 did not decrease the number of cell aggregates (not shown), although cell morphology became difficult to evaluate because of overgrowth of the cultures during these longer experiments.

The observation that N-CAM and L1 levels did not decline for 24 h after the withdrawal of hOP-1 suggested that hOP-1 might transduce a long-lasting signal that regulates the expression of N-CAM and L1. To test this possibility, NG108-15 cells were incubated for 1–24 h with 40 ng/ml hOP-1, washed twice to remove any residual hOP-1, and incubated for a total of 24 h in the absence of hOP-1. Incubation of NG108-15 cells with hOP-1 for 1 or 2 h did not significantly increase IgCAM expression after 24 h. In contrast, 4 h of treatment with hOP-1 (followed by 20 h without hOP-1) produced the same induction of N-CAM as did 24 h of treatment with hOP-1 (Fig. 7c). These data indicate that 4 h of treatment with hOP-1 triggers a cellular event that commits the cell to increased expression of IgCAMs.

Effect of hOP-1 on the Stability of N-CAM and L1 mRNA—hOP-1 could regulate IgCAM gene expression by stimulating gene transcription or increasing mRNA stability. If hOP-1 increases mRNA stability, then treatment of NG108-15 cells with actinomycin D, which blocks DNA transcription, should not diminish levels of N-CAM and L1 mRNA in hOP-1-treated cells. Accordingly, NG108-15 cells were incubated for 6 h in the absence and presence of 40 ng/ml hOP-1 and 0.5 µg/ml actinomycin D. This exposure to actinomycin D inhibited by more than 95% the uptake of [³H]uridine into an acid-insoluble pool (not shown) but did not affect cell viability. Treatment of cells with hOP-1 for 6 h increased levels of N-CAM and L1 mRNA, and this effect was completely blocked by actinomycin D (Fig. 8). These findings indicate that hOP-
FIG. 6. Kinetics of hOP-1 regulation of N-CAM and L1 protein and mRNA. NG108-15 cells were cultured in the absence and presence of 40 ng/ml hOP-1. The cells were harvested after the indicated number of hours and processed for Western (a) and Northern (b) blot analysis using 60 μg of protein and 20 μg of total RNA, respectively. Immunoblots were probed with mAbs H28.123 and 74-5H7 against N-CAM and L1, respectively. Hybridization of RNA was performed at 42 °C with a human cDNA probe NIL6 coding for N-CAM-140 and a full-length human L1 cDNA probe, each labeled with [32P]dCTP using random primers (see “Experimental Procedures”). The positions of RNA ladders are shown next to the Northern blot for N-CAM and do not coincide with the L1 blot. The size of the L1 transcript was estimated to be approximately 6.0 kb. c, densitometric analysis of the autoradiographs shown above. The experiment was repeated three times with similar results.
OP-1 Regulates L1 and N-CAM Gene Expression

hOP-1 induced a large, concentration-dependent increase in the expression of the immunoglobulin cell adhesion molecules L1 and N-CAM. These changes were preceded or accompanied by large increases in the abundance of L1 and N-CAM mRNA, implying that hOP-1 regulates IgCAM levels by increasing IgCAM gene expression. The fact that increases in protein and mRNA were not always equal may indicate that hOP-1 also regulates the translation and degradation of IgCAMs. hOP-1 appears to increase gene transcription rather than message stability, because actinomycin D prevented the increases in L1 and N-CAM mRNA. hOP-1 is the first described growth factor that concurrently regulates L1 and N-CAM gene expression. It will be interesting to learn whether OP-1 or a homologue is responsible for the coordinate regulation of N-CAM and L1 gene expression during neural development and regeneration (22).

The major isoforms of N-CAM are encoded by the first 14 exons of the N-CAM gene and different combinations of exons 15–19 (8). Analysis of the 5′ region of the N-CAM gene suggests that a common regulatory unit with binding sites for several transcription factors promotes the synthesis of all RNA classes (9, 45). The observation that hOP-1 increases all detectable N-CAM transcripts implies that hOP-1 regulates N-CAM transcription from this common proximal element. In contrast, nerve growth factor selectively induces the 7.2-kb N-CAM transcript in PC12 cells, indicating that some factors may regulate a single splice variant of the N-CAM gene (15). N-CAM gene regulation also differs between mesenchymal and neural cells. TGF-β transcriptionally regulates N-CAM gene expression in 3T3 cells and embryo-derived fibroblasts but not in N2A neuroblastoma (23) or NG108-15 cells.²

The signaling process initiated by hOP-1 was slow, both in its onset and termination. At least 4 h of treatment with hOP-1 was required to trigger an increase in IgCAM expression that persisted over the ensuing 20 h. Once activated by hOP-1, this process sustained heightened IgCAM gene expression for at least 24 h after hOP-1 withdrawal and then subsided gradually over an additional 2–3 days. A similarly slow activation of alkaline phosphatase activity has been reported after BMP-2 treatment of a bone marrow stromal cell line (46). The persistent stimulation of IgCAM gene expression after the removal of hOP-1 does not result from residual hOP-1, because a similar wash procedure prevented IgCAM induction after a 2-h pulse with hOP-1. These long latency responses suggest that IgCAM gene expression is triggered by the accumulation of a critical concentration of an autocrine substance or transcription factor. Evidence for an autocrine mechanism of IgCAM induction has been presented by Roubin and colleagues (23), who reported that conditioned medium from N2A neuroblastoma cells increases N-CAM expression in N2A cells or 3T3 fibroblasts (23). However, we found that N-CAM levels (per cell) did not change over a 20-fold range of NG108-15 cell densities, either in the absence or presence of hOP-1,² making it less likely that hOP-1 stimulated the production of an autocrine regulator of IgCAM expression in NG108-15 cells. Some bone morphogenetic proteins (BMP) induce the late phase expression of c-fos in bone (47), and, conceivably, OP-1 (BMP-7) acts similarly.

The signaling pathway for OP-1 is presently undefined. The failure of either forskolin or phorbol esters to increase the expression of N-CAM in NG108-15 cells suggests that hOP-1 does not regulate IgCAM expression by stimulating protein kinases A or C. Further evidence that cAMP is not a second messenger for hOP-1 was obtained in studies of osteoblast-enriched cultures, where hOP-1 fails to stimulate cAMP accumulation in the absence of parathyroid hormone (38). Several of the cloned TGF-ß receptors are serine-threonine kinases (48); homologous receptors may transduce the signals of the decapentaplegic-Vg1-related subfamily. It also appears that protein kinase A does not modulate the signal transduction pathway that mediates hOP-1 induction of IgCAMs in NG108-15 cells. A 50-fold increase in cAMP levels produced by forskolin did not modify the induction of N-CAM or L1 by hOP-1. Increased levels of cAMP do potentiate and inhibit, respectively, the smaller induction of N-CAM and L1 by nerve growth factor in PC12 cells (17).

The actions of hOP-1 were specific and independent of cell differentiation. hOP-1 did not regulate the expression of Thy-1 (another member of the immunoglobulin superfamily), N-cadherin (a calcium-dependent CAM), two neuron-specific cytoskeletal proteins (tau and neurofilament-200), or the ß subunit of the stimulatory GTP binding protein Gß. In contrast to the effects of nerve growth factor in PC12 cells (15, 19) and retinoic acid in F19 neuronal cells (20), hOP-1 regulated the expression of N-CAM and L1 without inducing differentiation. hOP-1 did not decrease cell proliferation and caused only a small proportion of cells to extend neurites. Moreover, hOP-1 increased L1 and N-CAM gene expression within 6 h, well before the cells exhibited morphological changes. Finally, Me2SO and forskolin, while more effective than hOP-1 in promoting neurite outgrowth, did not regulate IgCAM expression or modulate the effects of hOP-1.

Up-regulation of N-CAM and L1 increased the adhesiveness of hOP-1-treated NG108-15 cells and led to striking morphological changes, including the formation of epithelioid sheets and multilayered cell aggregates. This morphology likely evolved as post-mitotic, hOP-1-treated NG108-15 cells adhered to each other and nearby cells, forming increasingly large cell aggregates after each round of cell division. The adhesiveness of hOP-1-treated cells was reduced about 50% by antibodies against N-CAM (30) or L1; hence, it is possible that N-CAM and L1 act coordinately to increase NG108-15 cell adhesiveness and change the morphology of proliferating cells.

Cell-cell contact increases the expression of L1 in PC12 cells (19); however, it seems likely that the aggregation of hOP-1-treated NG108-15 cells was the consequence, rather than the cause of increased IgCAM expression. Cell-cell contact did not increase significantly until 1–2 days after treatment with hOP-1, whereas clear increases in L1 and N-CAM mRNA were apparent within the first 6–12 h. Furthermore, hOP-1-treated NG108-15 cells remained clumped after the removal of hOP-1, even after N-CAM and L1 levels had returned to control values. The persistence of cell aggregation after the loss of N-CAM and L1 suggests that cytoskeletal or membrane modifications may strengthen the intercellular contacts established by IgCAMs in NG108-15 cells.

The OP-1 precursor molecule and hOP-1 exhibited equivalent IgCAM-inducing activity in NG108-15 cells. This observation suggests that NG108-15 cells express proteases that activate pOP-1 or bear receptors that can transduce equally well the signals carried by hOP-1 and pOP-1. Cell-specific activation of the precursor form of TGF-ß has been described in co-cultures of endothelial and smooth muscle cells (49) and in EIA-transformed 293 cells (50). Since TGF-ß receptors are present on most cell types (43), the selective expression of activating proteases may serve to narrow the range of TGF-ß-responsive cells (50). A similar mechanism may constrain the actions of OP-1.

OP-1 is expressed in mouse brain during development (E17, P3) and adulthood (9 months old) (28, 29). This developmental profile is consistent with our hypothesis that OP-1 is a physiological regulator of IgCAMs. However, it is important to note that other members of the decapentaplegic-Vg1-related subfamily are dynamically expressed in brain during development, and some of these reproduce the effects of OP-1 in bone (26). Indeed, the evolutionarily conserved subfamily of bone morphogenetic proteins BMP-2 and BMP-4 more potently induces IgCAMs than the subfamily comprising BMP-5, BMP-6, and OP-1 (BMP-7), whereas the TGF-ß subfamily is inactive. Further work will define the role of these morphogens in the regulation of nervous system development and regeneration.

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REFERENCES
OP-1 Regulates L1 and N-CAM Gene Expression