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SH2B1β (SH2-Bβ) Enhances Expression of a Subset of Nerve Growth Factor-Regulated Genes Important for Neuronal Differentiation Including Genes Encoding Urokinase Plasminogen Activator Receptor and Matrix Metalloproteinase 3/10

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Previous work showed that the adapter protein SH2B adapter protein 1β (SH2B1) (SH2-B) binds to the activated form of the nerve growth factor (NGF) receptor TrkA and is critical for both NGF-dependent neurite outgrowth and maintenance. To identify SH2B1β-regulated genes critical for neurite outgrowth, we performed microarray analysis of control PC12 cells and PC12 cells stably overexpressing SH2B1β (PC12-SH2B1β) or the dominant-negative SH2B1β(R555E) [PC12-SH2B1β(R555E)]. NGF-induced microarray expression of Plaur and Mmp10 genes was greatly enhanced in PC12-SH2B1β cells, whereas NGF-induced Plaur and Mmp3 expression was substantially depressed in PC12-SH2B1β(R555E) cells. Plaur, Mmp3, and Mmp10 are among the 12 genes most highly upregulated after 6 h of NGF. Their protein products [urokinase plasminogen activator receptor (uPAR), matrix metalloproteinase 3 (MMP3), and MMP10] lie in the same pathway of extracellular matrix degradation; uPAR has been shown previously to be critical for NGF-induced neurite outgrowth. Quantitative real-time PCR analysis revealed SH2B1β enhancement of NGF induction of all three genes and the suppression of NGF induction of all three when endogenous SH2B1 was reduced using short hairpin RNA against SH2B1 and in PC12-SH2B1β(R555E) cells. NGF-induced levels of uPAR and MMP3/10 and neurite outgrowth through Matrigel (MMP3-dependent) were also increased in PC12-SH2B1β cells. These results suggest that SH2B1β stimulates NGF-induced neuronal differentiation at least in part by enhancing expression of a specific subset of NGF-sensitive genes, including Plaur, Mmp3, and/or Mmp10, required for neurite outgrowth. (*Molecular Endocrinology* 22: 454–476, 2008)

NERVE GROWTH FACTOR (NGF) plays a critical role in the development of the peripheral nervous system. This is especially true for sympathetic neurons, which are overproduced during development and are believed to compete for the limited quantities of target-derived NGF for their survival; only the select neurons that successfully innervate NGF-producing target organs persist (1–3). Sympathetic neuron requirement for NGF for survival has been well documented both *in vitro* and *in vivo* (reviewed in Refs. 4 and 5). However, due to the absolute NGF dependence of sympathetic neurons for survival, it has been difficult to assess the necessity for NGF on other aspects of sympathetic neuron development and function, such as axonal growth and target innervation. The use of *Bax* knockout mice circumvented this problem. Deletion of *Bax*, a proapoptotic transcription factor, dramatically reduces trophic-factor deprivation-induced apoptosis of both sensory and sympathetic neurons (6). The sensory and sympathetic neurons that die off in mice lacking NGF ($NGF^{-/-}$) and the NGF receptor TrkA ($TrkA^{-/-}$) (7, 8) are able to persist when those mice are crossed with the $Bax^{-/-}$ mice (9). In the double knockout mice, axons from both sympathetic and sensory neurons appear unable to reach their targets, resulting in impaired target innervation (9–11). Although experiments using these mice implicate NGF as an essential signal required for normal axonal growth of and peripheral target innervation by sympathetic and sensory neurons *in vivo*, the actual mechanisms by which NGF elicits these effects are only

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Abbreviations: ECM, Extracellular matrix; EGF, epidermal growth factor; EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDNF, glial cell line-derived neurotrophic factor; GFP, green fluorescent protein; MMP, matrix metalloproteinase; NGF, nerve growth factor; QT-PCR, real-time quantitative PCR; RNAi, RNA interference; SH2B1, SH2B adapter protein 1β; shRNA, short hairpin RNA; siRNA, small interfering RNA; uPAR, urokinase plasminogen activator receptor.

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beginning to be understood. Most of what we know about NGF-induced neuronal differentiation and NGF signal transduction events has come from the PC12 cell model. PC12 cells, derived from a rat pheochromocytoma, can be induced to differentiate into sympathetic neuron-like cells by NGF (12, 13). Upon exposure to NGF, these cells first cease to proliferate. They then exhibit somatic hypertrophy, acquire neurites, and express neuronal-specific genes. The neurite outgrowths are capable of forming typical looking synapses with primary neurons from rat cortex (14). As has been documented for sympathetic and sensory neurons, once PC12 cells differentiate into a neuronal phenotype, they depend on NGF for survival (for review, see Ref. 15).

NGF initiation of neuronal differentiation starts with NGF binding to its receptor tyrosine kinase TrkA and activating TrkA. The activated TrkA autophosphorylates, and the phosphorylated tyrosines in the TrkA cytoplasmic domain bind a number of different signaling molecules and initiate a variety of downstream signaling pathways implicated in neuronal differentiation (16, 17). Binding proteins include Shc, phospholipase C-γ (PLC-γ), fibroblast growth receptor substrate 2 (Frs2, SNT), and CHK, a homolog of the cytoplasmic tyrosine kinase CSK (control of Src kinase). Binding of these and presumably other TrkA binding proteins lead to the activation of phospholipase C-γ/Ca^{2+}, nuclear factor-κB, phosphatidylinositol-3-kinase/Akt, Ras/Raf/MEK/ERK1 and -2; Jun N-terminal kinase, p38, and atypical protein kinase C pathways (reviewed in Refs. 18 and 19).

We and others identified the putative adapter/scaffold protein SH2B adapter protein 1β (SH2B1) as one of the signaling proteins that bind to the NGF-activated form of TrkA (20, 21). NGF promotes the rapid association of SH2B1 with TrkA and subsequent phosphorylation of SH2B1 on tyrosines as well as serines/threonines (20–22). SH2B1 belongs to a family of adapter proteins that also includes APS (SH2B2) and Lnk (SH2B3) (23–25). SH2B1 family members contain a pleckstrin homology domain, at least one dimerization domain, and a C-terminal SH2 domain (reviewed in Ref. 26); it is the SH2 domain that binds to TrkA. The four known SH2B1 splice variants, α, β, γ, and δ, differ only in their C termini starting just past the SH2 domain (21, 27, 28), suggesting that all isoforms would be recruited to TrkA and would share many, but perhaps not all, responses. SH2B1 has been implicated in NGF-induced neurite outgrowth of PC12 cells (20, 21). Thus, overexpression of SH2B1α or SH2B1β enhances NGF-induced neurite outgrowth in PC12 cells. In contrast, mutating a critical arginine [SH2B1β(R555E)] within the SH2 domain of SH2B1β that prevents SH2B1β from binding to and being phosphorylated by TrkA blocks NGF-induced neurite outgrowth of PC12 cells (21, 22). SH2B1 has also been implicated in the NGF-dependent maintenance of explants of sympathetic cervical ganglia (20). Dissociated primary sympathetic neurons growing in NGF-containing medium exhibit a reduced rate of survival when anti-SH2B1 antibodies are introduced by trituration. Similarly, axonal processes are nearly eliminated when an SH2B1 mutant that blocks SH2B1-mediated signaling is introduced within explants of sympathetic ganglia grown in the presence of NGF. In contrast, NGF-treated neurons into which wild-type SH2B1 is introduced thrive and have elaborate, long-branching axonal processes.

The mechanism by which SH2B1 enhances neurite outgrowth is unknown. Qian and Ginty (29) suggest that SH2B1 potentiates NGF induction of autophosphorylation of TrkA, assessed using an antibody to phosphotyrosine 490 in TrkA, the binding site for Shc. Such a generalized stimulation of TrkA activity by SH2B1 might be expected to enhance the expression of all NGF-sensitive genes. However, SH2B1 potentiation of NGF induction of TrkA activity cannot explain the ability of the dominant-negative SH2B1β(R555E) to block NGF-induced neurite outgrowth, because PC12-SH2B1β(R555E) cells did not exhibit decreased TrkA autophosphorylation in general (21) or on tyrosine 490 (Maures, T., and C. Carter-Su, unpublished observation) in response to NGF. The inability of SH2B1β(R555E) to inhibit NGF-induced tyrosyl phosphorylation of TrkA, phospholipase Cγ, or Shc or activation of ERKs 1 and 2 led us to hypothesize that SH2B1β may initiate a previously unknown NGF signaling pathway (21). SH2B1β contains both a nuclear export (30) and nuclear localization signal (Maures, T., L. Chen, and C. Carter-Su, manuscript in preparation). Experiments using truncated and mutated forms of SH2B1β, as well as the inhibitor of nuclear export leptomycin B, revealed that both ectopically expressed and endogenous SH2B1β shuttle between the cytoplasm/plasma membrane and the nucleus (30). Unlike wild-type SH2B1β, SH2B1β mutants with a defective nuclear export (30) or import (Maures, T., L. Chen, and C. Carter-Su, manuscript in preparation) signal are unable to enhance NGF-induced neurite outgrowth. Together, these findings led us to hypothesize that whereas SH2B1β might have a modest stimulatory effect on multiple NGF-sensitive genes, there would exist a specific subset of genes important for neurite outgrowth that were particularly sensitive to SH2B1β.

To determine whether SH2B1β regulates all or a subset of NGF-responsive genes early in the NGF-induced neuronal differentiation program, and at the same time identify SH2B1-regulated genes important for neurite outgrowth, we performed microarray analysis on cRNA prepared from NGF-treated PC12 cells stably expressing SH2B1β, SH2B1β(R555E), or SH2B1β vector. We identified both a subset of NGF-responsive genes whose NGF-regulated expression is enhanced substantially further by SH2B1β and/or substantially dampened by SH2B1β(R555E) as well as a subset of genes whose expression is not altered by SH2B1β or SH2B1β(R555E). We confirmed the NGF and SH2B1β regulation of a subset of these SH2B1β-regulated genes using quantitative real-time PCR (QT-PCR). We further characterized SH2B1β regulation of

the proteins encoded by three of the genes most highly up-regulated by NGF and whose NGF up-regulation appeared to depend upon SH2B1β: Plaur, Mmp3, and Mmp10, encoding urokinase plasminogen activator receptor (uPAR), matrix metalloproteinase (MMP)-3 (stromelysin-1; transin-1), and MMP10 (stromelysin-2; transin-2), respectively. These proteins were of particular interest because uPAR has been shown previously to be required for NGF-induced neuronal differentiation, and MMP3 and MMP10 lie in the same extracellular matrix (ECM) degradation pathway as uPAR. Consistent with MMP3 being important for neurite outgrowth and penetration through an ECM, we showed that SH2B1β enhances neurite extension through Matrigel. Finally, we confirmed a critical role for endogenous SH2B1 in the expression of these proteins using short hairpin RNA (shRNA) to SH2B1, which was found to suppress NGF-induced expression of Plaur, Mmp3, and Mmp10.

RESULTS

Identification of a Subset of NGF-Responsive Genes that Are Regulated by SH2B1β

To determine whether SH2B1β regulates the expression of all or a subset of NGF-sensitive genes in cells and at the same time identify SH2B1-regulated genes important for neurite outgrowth, we performed a global analysis of gene expression in pooled PC12 cells stably expressing green fluorescent protein (GFP) (control cells), GFP-tagged wild-type SH2B1β (PC12-SH2B1β cells) or GFP-tagged dominant-negative SH2B1β(R555E) [PC12-SH2B1β(R555E) cells] treated with or without 100 ng/ml NGF for 6 h. Microarray analysis of control PC12 cells using the rat Affymetrix gene chip RAE223A that detects about 15,900 known genes and expressed sequence tags (ESTs) identified 511 genes and ESTs whose expression at least doubled in response to NGF (Fig. 1 and supplemental table S1, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). Expression of 79 known genes more than tripled (Table 1). Microarray analysis of control PC12 cells identified a similar number (513) of genes whose expression was decreased by more than 50% after NGF treatment (Fig. 1 and supplemental Table S2). Together, these microarray studies indicate that NGF rapidly regulates (both positively and negatively) the expression of about 1000 genes/ESTs, many more than previously recognized.

Among the 511 genes/ESTs whose expression was at least doubled by NGF in control cells, 34 of them showed a further doubling in NGF induction in PC12-SH2B1β cells (Fig. 1 and Table 2), whereas 153 of them displayed a 50% or greater reduction in NGF-dependent expression in PC12-SH2B1β(R555E) cells (Fig. 1). Only two known genes (Plaur and Glrx1) and four ESTs fit both criteria [*i.e.* exhibit NGF induction that was both enhanced by at least 100% in PC12-SH2B1β cells and reduced by 50% or more in PC12-SH2B1β(R555E) cells] (Fig. 1 and Table 3).

Similarly, among the 513 genes/ESTs whose expression was down-regulated by NGF by at least 50%, 49 showed a substantially greater (at least a doubling of the NGF inhibition seen in control cells) NGF-dependent reduction in expression in PC12-SH2B1β cells (Fig. 1 and Table 4), whereas 105 exhibited a substantially diminished (by ≥50%) NGF-dependent decrease in expression in PC12-SH2B1β(R555E) cells (supplemental Table S4). None of the known genes and only one EST fulfilled both of these criteria. The finding that some, but not all, of the NGF-responsive genes show enhanced responsiveness to NGF in SH2B1β-overexpressing cells and/or reduced responsiveness to NGF in SH2B1β(R555E)-expressing cells is consistent with SH2B1β enhancing the NGF-induced regulation of expression of a subset of NGF-responsive genes during neuronal differentiation.

Functional Classification of NGF-Responsive Genes that Are Also Regulated by SH2B1β

To obtain insight into the cellular processes that are regulated by SH2B1β, genes whose expression level was induced or inhibited by greater than a factor of two after treatment with NGF were grouped into gene ontology categories using DAVID (database for annotation, visualization, and integrated discovery) (31). For each of the three different cell lines, an average of 76% of input genes were categorized. As predicted given NGF's ability to promote neuronal differentiation, after stimulation of control PC12 cells with NGF for 6 h, the expression of genes associated with cell differentiation, intracellular signaling cascade, cell death, organogenesis, and morphogenesis was enhanced (Fig. 2A). Compared with control cells, PC12-SH2B1β cells had an increased number of NGF-induced genes overrepresented in all of the above-mentioned categories, whereas PC12-SH2B1β(R555E) cells had a decreased number. Also as predicted from NGF's ability to promote neuronal differentiation, genes whose expression in control PC12 cells was decreased by NGF by more than 50% were associated with cell cycle, cell proliferation, and cell migration (Fig. 2B). Overexpressing SH2B1β increased the number of NGF-induced genes overrepresented in all of the above-mentioned categories, whereas overexpressing SH2B1β(R555E) reduced NGF-induced overrepresentation of genes in all three categories. In addition, genes in the category of cell differentiation were overrepresented in cells overexpressing SH2B1β and to a lesser extent in cells overexpressing SH2B1β(R555E). These findings are consistent with SH2B1β increasing NGF-induced neuronal differentiation at least in part by enhancing NGF-induced expression of a subset of genes whose products are implicated in morphogenesis, organogenesis, cell survival, and intracellular signaling and by increasing the degree of NGF inhibition of expression of a

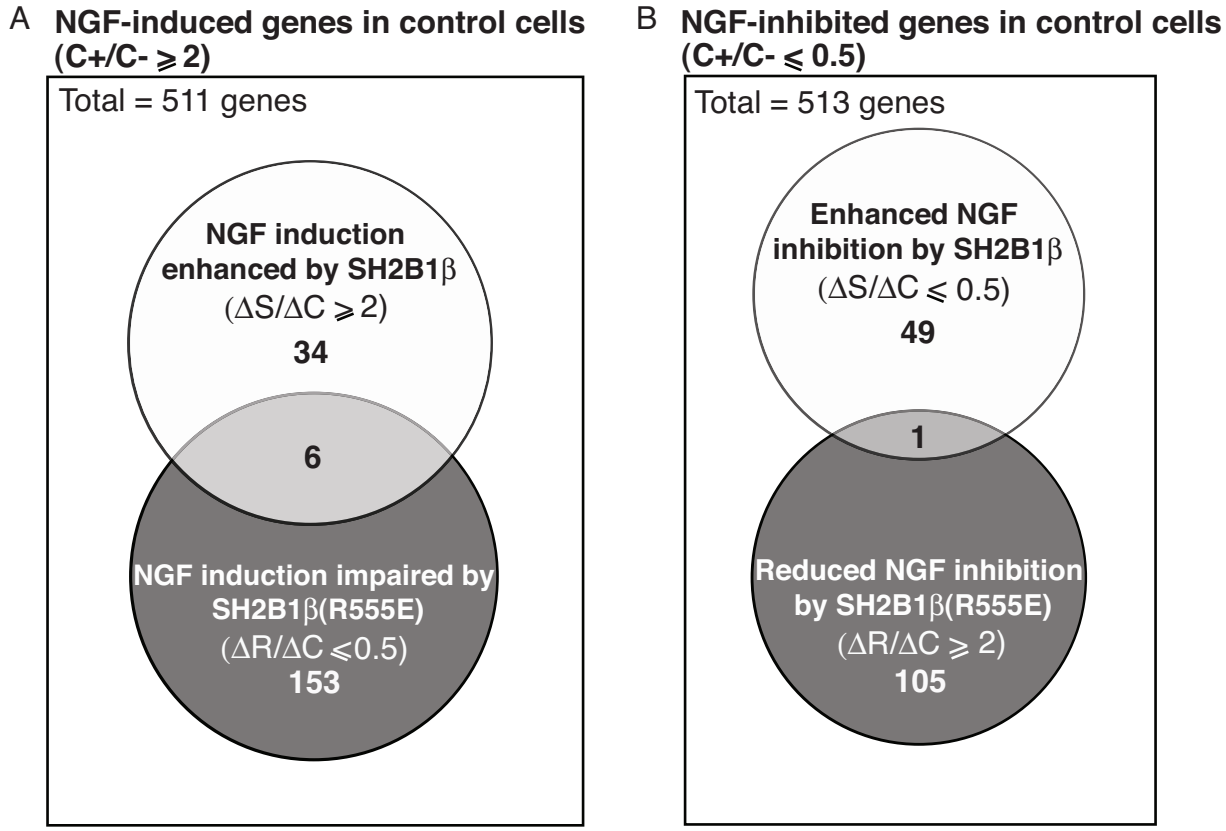


Fig. 1. SH2B1β Enhances a Subset of NGF-Responsive Genes

A, The expression of 511 genes and ESTs (588 probe sets) was at least doubled by treatment of control PC12 cells with NGF (6 h, 100 ng/ml) (C+/C− ≥ 2). Among the 511 genes and ESTs, the NGF-induced expression of 34 genes and ESTs was enhanced by at least 100% by overexpression of SH2B1β, represented by the *white circle* (C+/C− ≥ 2 and ΔS/ΔC ≥ 2 where ΔS = S+/S− and ΔC = C+/C−). Among the 511 genes and ESTs, the NGF-induced expression of 153 genes was reduced by 50% or more in cells overexpressing SH2B1β(R555E), represented by the *dark gray circle* (C+/C− ≥ 2 and ΔR/ΔC ≤ 0.5 where ΔR = R+/R− and ΔC = C+/C−). There were only two genes and four ESTs that matched both criteria, represented by the *overlapping area between the white and dark gray circles* (C+/C− ≥ 2; ΔS/ΔC ≥ 2; ΔR/ΔC ≤ 0.5). B, The expression of 513 genes and ESTs (507 probe sets) was reduced by at least 50% by treatment with NGF (6 h, 100 ng/ml) (C+/C− ≤ 0.5). Among the 513 genes and ESTs, the expression of 49 genes and ESTs was further reduced by overexpression of SH2B1β, represented by the *white circle* (C+/C− ≤ 0.5 and ΔS/ΔC ≤ 0.5). The NGF-induced inhibition of 105 genes and ESTs was, in addition, dampened by overexpression of SH2B1β(R555E), represented by the *dark gray circle* (C+/C− ≤ 0.5 and ΔR/ΔC ≥ 2). There was only one EST that matched both criteria, represented by the *overlapping area between the white and dark gray circles* (C+/C− ≤ 0.5; ΔS/ΔC ≤ 0.5; ΔR/ΔC ≥ 2). C−, S−, and R− represent gene expression levels in control PC12, PC12-SH2B1β, and PC12-SH2B1β(R555E) cells, respectively, in the absence of NGF. C+, S+ and R+ represent gene expression levels in NGF-treated control PC12, PC12-SH2B1β, and PC12-SH2B1β(R555E) cells, respectively. ΔC = C+/C−; ΔS = S+/S−; ΔR = R+/R−.

subset of genes whose products are implicated in cell cycle progression, cell proliferation, and cell migration. SH2B1β(R555E) appears to play a dominant-negative role with regard to a subset of NGF-regulated genes that affect morphogenesis, organogenesis, cell survival, intracellular signaling, and cell differentiation, consistent with the substantially decreased ability of PC12-SH2B1β(R555E) cells to differentiate into a neuronal phenotype in response to NGF.

Novel SH2B1β-Regulated Genes Likely to Be Involved in Neuronal Differentiation

Because PC12-SH2B1β and PC12-SH2B1β(R555E) cells display substantially enhanced and impaired neurite outgrowth, respectively, we reasoned that genes whose expression pattern is substantially enhanced after NGF treatment and whose NGF enhancement is greatly increased in PC12-SH2B1β cells and/or substantially decreased in PC12-SH2B1β(R555E) cells would be prime candidates for further investigation. Seventeen known genes exhibited at least a doubling of expression in response to NGF in control cells and at least a further doubling in NGF-induced expression in PC12-SH2B1β cells (Table 2). Of these 17 genes, Mmp10 exhibited both the greatest NGF induction of gene expression in control PC12 cells (+NGF/−NGF ~38) and the greatest further enhancement (approximately seven times) upon overexpression of SH2B1β. Mmp10 encodes MMP10, a proteinase involved in the

Table 1. NGF Induces Increases in Gene Expression in PC12 Cells

Gene Symbol	Gene Title	Unigene	C+/C−
Mmp3	MMP3	Rn.32086	114.81
Crh	CRH	Rn.10349	56.80
Pai1	Serine (or cysteine) proteinase inhibitor, member 1	Rn.29367	46.58
Mmp10	MMP10	Rn.9946	38.02
Mmp13	MMP13	Rn.10997	22.90
Egr1	Early growth response 1	Rn.9096	21.60
Ca2[a]	Carbonic anhydrase 2	Rn.26083	14.81
Scn6a	Sodium channel, voltage-gated, type 6, α-polypeptide	Rn.54541	12.32
Fosl1	Fos-like antigen 1	Rn.11306	10.22
Plaur	Plasminogen activator, urokinase receptor	Rn.82711	9.78
Vrl1	Vanilloid receptor-like protein 1	Rn.44866	7.73
Vgf	VGF NGF-inducible	Rn.9704	7.15
Krt1–18	Keratin complex 1, acidic, gene 18	Rn.11104	6.78
Ret[a]	Ret protooncogene	Rn.44178	6.66
Dusp6	Dual-specificity phosphatase 6	Rn.4313	6.61
Ania4	Activity and neurotransmitter-induced early gene protein 4	Rn.40517	6.41
Klf5	Kruppel-like factor 5 (intestinal)	Rn.17478	5.50
Bmp1	Bone morphogenetic protein 1 (procollagen C-proetinase)	Rn.87080	5.43
Giot2	Gonadotropin-inducible ovarian transcription factor 2	Rn.77686	5.19
Nid67	Putative small membrane protein NID67	Rn.8865	5.18
Glrx1[a]	Glutaredoxin 1 (thioltransferase)	Rn.1484	4.94
Ifrd1	Interferon-related developmental regulator 1	Rn.3723	4.90
LOC286921	Aldose reductase-like protein	Rn.23676	4.83
Tage4	Tumor-associated glycoprotein pE4	Rn.10677	4.83
Tjp2	Tight junction protein 2	Rn.10965	4.70
Gpr37	G protein-coupled receptor 37(endothelin receptor type B-like)	Rn.28035	4.69
Tub	Tubby (mouse) homolog	Rn.30017	4.66
Tfpi	Tissue factor pathway inhibitor	Rn.15795	4.56
Prkaa2	AMP-activated protein kinase	Rn.64583	4.48
Pcscl	Peroxisomal Ca-dependent solute carrier-like protein	Rn.17644	4.47
Cga	Glycoprotein hormones, α-subunit	Rn.10589	4.22
Ctsk	Cathepsin K	Rn.5598	4.20
Ager	Advanced glycosylation end product-specific receptor	Rn.9829	4.10
Scya2	Small inducible cytokine A2	Rn.4772	4.10
Fgf15	Fibroblast growth factor 15	Rn.81230	4.04
Arc	Activity-regulated cytoskeletal-associated protein	Rn.10086	3.98
Lnk	Linker of T-cell receptor pathways	Rn.11228	3.98
Bat3[a]	HLA-B-associated transcript 3	Rn.22304	3.97
Itga1	Integrin, α1	Rn.91044	3.87
Hes1	Hairy and enhancer of split 1 (*Drosophila*)	Rn.19727	3.87
Ptafr	Platelet-activating factor receptor	Rn.10137	3.80
Lgals3	Lectin, galactose binding, soluble 3	Rn.764	3.79
Rgs5	Regulator of G-protein signaling 5	Rn.1150	3.78
Glrx1	Glutaredoxin 1 (thioltransferase)	Rn.1484	3.77
Synd1	Syndecan	Rn.11176	3.74
S100a10	S-100-related protein, clone 42C	Rn.4083	3.69
Ak4	Adenylate kinase 4	Rn.44288	3.68
Scamp5	Secretory carrier membrane protein 5	Rn.24420	3.66
Rdc1	Chemokine orphan receptor 1	Rn.12959	3.65
Bdnf[a]	Brain-derived neurothrophic factor	Rn.11266	3.59
Filip	Filamin-interacting protein L-Filip	Rn.44931	3.59
Cdkn1a	Cyclin-dependent kinase inhibitor 1A	Rn.10089	3.57
Ptgs1	Prostaglandin-endoperoxide synthase 1	Rn.44404	3.51
Hpx	Hemopexin	Rn.2380	3.50
Ecel1	Endothelin converting enzyme-like 1	Rn.45803	3.47
Hk2	Hexokinase 2	Rn.91375	3.44
Clcn2	Chloride channel 2	Rn.11073	3.39
Upb1	Ureidopropionase, β	Rn.11110	3.38
Snk	Serum-inducible kinase	Rn.12100	3.38
Nr2f6	Nuclear receptor subfamily 2, group F, member 6	Rn.25840	3.35
Junb	Jun B protooncogene	Rn.15806	3.32
Prss11	Protease, serine, 11	Rn.2782	3.32

(*Table continues*)

Table 1. *Continued*

Gene Symbol	Gene Title	Unigene	C+/C−
Mmp3	MMP3	Rn.32086	114.81
Adcyap1r1	Adenylate cyclase activating polypeptide 1 receptor 1	Rn.88408	3.32
Lasp1	LIM and SH3 protein 1	Rn.69815	3.30
Ehd4	Pincher	Rn.7379	3.29
Msg1	Melanocyte-specific gene 1 protein	Rn.8163	3.26
Gls	Glutaminase	Rn.5762	3.23
Axcam	Axonal-associated cell adhesion molecule	Rn.10117	3.17
Dcamkl1	Double cortin and calcium/calmodulin-dependent protein kinase-like 1	Rn.80575	3.16
Stc1	Stanniocalcin 1	Rn.10647	3.12
Cyp4a12	Cytochrome P450, 4a12	Rn.10034	3.08
S100a4	S100 calcium-binding protein A4	Rn.504	3.06
LOC59314	CaM-KII inhibitory protein	Rn.42880	3.06
Slc6a2	Solute carrier family 6(neurotransmitter transporter, noradrenalin),member 2	Rn.14577	3.05
Thrsp	Thyroid hormone responsive protein (spot14)	Rn.81140	3.04
Tceb3	Transcription elongation factor B (SIII), polypeptide 3 (110 kDa)	Rn.37427	3.04
Anxa2	Calpactin I heavy chain	Rn.28	3.04
Crmp4	Collapsin response mediator protein 4	Rn.8499	3.01

Control PC12 cells were incubated for 0 or 6 h with 100 ng/ml NGF, and gene expression profiles were assessed as described in *Materials and Methods*. Gene symbols, gene titles, and unigene numbers are listed for the 77 known genes exhibiting NGF-induced gene expression of at least three times basal (no NGF) in control PC12 cells (C+/C− ≥ 3), arranged in descending order based on the magnitude of the NGF induction of expression. The 74 ESTs that met this criterion are included in supplemental table S1. C− and C+ indicate gene expression levels in untreated and NGF-treated control PC12 cells, respectively.

[a] Genes represented by more than one probe set; values listed are the average of the replicates.

degradation of the ECM (reviewed in Ref. 32). Among these same 17 genes, only Plaur and Glrx1 (highlighted in Table 2) also exhibited a significantly reduced (≥50% reduction) response to NGF in PC12-SH2B1β(R555E) cells compared with control cells. Plaur and Glrx1 encode uPAR and glutaredoxin 1, respectively. Plaur was of particular interest because of its being identified previously as a primary response gene for NGF critical for

Table 2. NGF-Responsive Genes Whose Expression Is Enhanced by SH2B1b

Gene Symbol	Gene Title	Unigene	C+/C−	S+/S−	R+/R−	**ΔS/ΔC**	ΔR/ΔC
Mmp10	MMP10	Rn.9946	38.02	271.78	45.24	**7.15**	1.19
Stc1	Stanniocalcin 1	Rn.10647	3.12	20.87	2.05	**6.69**	0.66
Plaur	Plasminogen activator, urokinase receptor	Rn.82711	9.78	56.16	4.44	**5.74**	0.45
Scn6a	Sodium channel, voltage-gated, type 6, α polypeptide	Rn.54541	12.32	65.40	23.36	**5.31**	1.90
Ca2[a]	Carbonic anhydrase 2	Rn.26083	14.81	76.20	7.90	**5.13**	0.55
Fosl1	Fos-like antigen 1	Rn.11306	10.22	48.84	5.48	**4.78**	0.54
Arc	Activity-regulated cytoskeletal-associated protein	Rn.10086	3.98	14.44	6.85	**3.62**	1.72
Glrx1[a]	Glutaredoxin 1 (thioltransferase)	Rn.1484	4.94	17.82	1.90	**3.57**	0.41
Dusp6	Dual-specificity phosphatase 6	Rn.4313	6.61	17.73	7.45	**2.68**	1.13
LOC286921	Aldose reductase-like protein	Rn.23676	4.83	12.78	4.80	**2.64**	0.99
Ania4	Activity- and neurotransmitter-induced early gene protein 4 (ania-4)	Rn.40517	6.41	14.77	7.42	**2.30**	1.16
Egr1	Early growth response 1	Rn.9096	21.60	49.52	27.71	**2.29**	1.28
Kzf2	KRAB-zinc finger protein KZF-2	Rn.10664	2.02	4.62	2.03	**2.29**	1.01
Tage4	Tumor-associated glycoprotein pE4	Rn.10677	4.83	10.70	4.29	**2.22**	0.89
Basp2	Brain abundant, membrane-attached signal protein 2	Rn.10928	2.23	4.64	1.40	**2.08**	0.63
Gprk5	G protein-coupled receptor kinase 5	Rn.6500	2.10	4.33	1.70	**2.06**	0.81
Bcl10	B-cell CLL/lymphoma 10	Rn.13007	2.20	4.51	2.03	**2.05**	0.92

Control PC12, PC12-SH2B1β, or PC12-SH2B1β(R555E) cells were incubated with vehicle or NGF for 6 h, and gene expression profiles were assessed as described in *Materials and Methods*. Gene symbols, gene titles, and unigene numbers are listed for the 17 known genes for which NGF induces at least a doubling of expression in control PC12 cells (C+/C− ≥ 2) and at least a further doubling of NGF-induced expression in PC12-SH2B1β (ΔS/ΔC ≥ 2), arranged in order of decreasing ability of SH2B1β to enhance NGF-induced gene expression. An additional 18 ESTs (unlisted) met this criterion. Two genes, Plaur and Glrx1 (and four ESTs not listed), additionally show a reduction in the magnitude of NGF induction of expression in PC12-SH2B1β(R555E) cells to a level that is less than 50% that seen with control PC12 cells (ΔR/ΔC ≤ 0.5). C+, C−, S+, S−, R+, R−, ΔC, ΔS, and ΔR are defined in Fig. 1. The sorting criterion of the table is designated in *bold*.

[a] Genes represented by more than one probe set; values listed are the average of the replicates.

Table 3. NGF-Responsive Genes Whose Expression Is Inhibited by SH2B1β(R555E)

Gene Symbol	Gene Title	Unigene	C+/C−	S+/S−	R+/R−	ΔS/ΔC	**ΔR/ΔC**
Pai1	Serine (or cysteine) proteinase inhibitor, member 1	Rn.29367	46.58	53.54	7.63	1.15	**0.16**
Vldlr	Very-low-density lipoprotein receptor	Rn.9975	2.25	2.35	0.65	1.04	**0.29**
Klf5	Kruppel-like factor 5 (intestinal)	Rn.17478	5.50	10.64	1.76	1.93	**0.32**
Mmp3	MMP3	Rn.32086	114.81	119.00	37.17	1.04	**0.32**
Pou2f1	POU domain, class 2, transcription factor 1	Rn.9992	2.24	3.38	0.78	1.51	**0.35**
Bcat1	Branched-chain aminotransferase 1, cytosolic	Rn.8273	2.73	3.72	1.00	1.36	**0.37**
Cryab	Crystallin, αB	Rn.54554	2.28	2.32	0.92	1.02	**0.40**
Ecel1	Endothelin converting enzyme-like 1	Rn.45803	3.47	5.57	1.43	1.61	**0.41**
Glrx1[a]	Glutaredoxin1 (thioltransferase)	Rn.1484	4.94	17.82	1.90	3.57	**0.41**
Msg1	Melanocyte-specific gene 1 protein	Rn.8163	3.26	3.46	1.36	1.06	**0.42**
TA1	Tumor-associated protein 1	Rn.32261	2.18	3.11	0.93	1.43	**0.42**
Nmrk	Non-MHC-restricted killing associated	Rn.53994	2.24	2.44	1.00	1.09	**0.45**
Plaur	Plasminogen activator, urokinase receptor	Rn.82711	9.78	56.16	4.44	5.74	**0.45**
Sv2b	Synaptic vesicle glycoprotein 2b	Rn.9940	2.30	2.84	1.07	1.23	**0.47**

Control PC12, PC12-SH2B1β, or PC12-SH2B1β(R555E) cells were incubated with vehicle or NGF for 6 h, and gene expression profiles were assessed as described in *Materials and Methods*. Gene symbols, gene titles, and unigene numbers are listed for the 14 known genes whose NGF-induced expression was at least doubled in control cells (C+/C− ≥ 2), greater in PC12-SH2B1β cells than in control PC12 cells (ΔS/ΔC > 1), and reduced in PC12-SH2B1β(R555E) cells to a level that is less than 50% that seen with control PC12 cells (ΔR/ΔC ≤ 0.5), arranged in order of decreasing ability of SH2B1β(R555E) to impair NGF-induced gene expression (decreasing ΔR/ΔC). An additional 11 ESTs (unlisted) met this criterion. C+, C−, S+, S−, R+, R−, ΔC, ΔS, and ΔR are defined in Fig. 1. The sorting criterion of the table is designated in *bold*.

NGF-induced neurite outgrowth and biochemical differentiation of PC12 cells (33, 34). Glutaredoxin 1 has not been previously shown to be up-regulated by NGF, much less by SH2B1, but was intriguing because it is found in neurons and modulates cellular redox status, important for cell survival against oxidative stress (35).

In addition, there were 12 genes whose NGF-induced level of expression in PC12-SH2B1β cells did not meet our criterion of being substantially greater (at least a doubling) than in control cells but whose NGF-induced expression was substantially reduced (by ≥50%) in PC12-SH2B1β(R555E) cells [1 < (S+/S−)/(C+/C−) < 2; (R+/R−)/(C+/C−) < 0.5] (Table 3). Among those genes, Mmp3 exhibited the greatest NGF induction of expression in control PC12 cells (+NGF/−NGF ~115) as well as one of the greatest reductions (68%) in NGF-stimulated expression in PC12-SH2B1β(R555E) cells. Mmp3 was of interest because it encodes MMP3, a proteolytic enzyme present in growth cones of NGF-treated PC12 cells and previously implicated in neurite penetration through the ECM (36).

NGF inhibits the expression of almost as many genes as it enhances the expression of, suggesting

Table 4. Genes Whose Expression Is Suppressed by NGF and Further Suppressed by SH2B1b

Gene Symbol	Gene Title	Unigene	C+/C−	S+/S−	R+/R−	**ΔS/ΔC**	ΔR/ΔC
Pola2	DNA polymerase α-subunit II	Rn.31773	0.44	0.10	0.30	**0.23**	0.69
Sord	Sorbitol dehydrogenase	Rn.11334	0.13	0.04	0.12	**0.28**	0.89
Gcgr	Glucagon receptor	Rn.11225	0.24	0.07	0.30	**0.31**	1.23
Prkwnk4	Protein kinase, lysine deficient 4	Rn.12652	0.49	0.17	0.82	**0.34**	1.66
Ga	Liver mitochondrial glutaminase	Rn.10202	0.47	0.16	0.37	**0.35**	0.80
Hip1	Huntingtin interacting protein 1	Rn.50506	0.47	0.17	0.59	**0.35**	1.24
Syt3	Synaptotagmin 3	Rn.48884	0.28	0.11	0.46	**0.38**	1.63
Rb2[a]	Retinoblastoma-related gene	Rn.11020	0.39	0.15	0.43	**0.39**	1.10
Cldn3	Claudin 3	Rn.4513	0.48	0.19	0.48	**0.40**	1.01
U91561	Pyridoxine 5-phosphate oxidase	Rn.6299	0.45	0.20	0.40	**0.44**	0.89
Rab3a	Ras-related small GTP binding protein 3A	Rn.44409	0.37	0.17	0.46	**0.46**	1.26
Pold1	DNA polymerase δ, catalytic subunit	Rn.88690	0.27	0.13	0.32	**0.48**	1.21

Control PC12, PC12-SH2B1β, or PC12-SH2B1β(R555E) cells were incubated with vehicle or 100 ng/ml NGF for 6 h, and gene expression profiles were assessed as described in *Materials and Methods*. Gene symbols, gene titles, and unigene numbers are listed for the 12 known genes whose expression in control cells is both reduced by NGF treatment to less than 50% of basal levels (C+/C− ≤ 0.5) and whose NGF-induced reduction is further reduced by at least an additional 50% in PC12-SH2B1β cells (C+/C− ≤ 0.5; ΔS/ΔC ≤ 0.5), arranged in the order of decreasing SH2B1β-dependent inhibition of gene expression (decreasing ΔS/ΔC). Thirty-four ESTs (not listed) fulfilled these criteria. C+, C−, S+, S−, R+, R−, ΔC, ΔS, and ΔR are defined in Fig. 1. The sorting criterion of the table is designated in *bold*.

[a] Genes represented by more than one probe set; values listed are the average of the replicates.

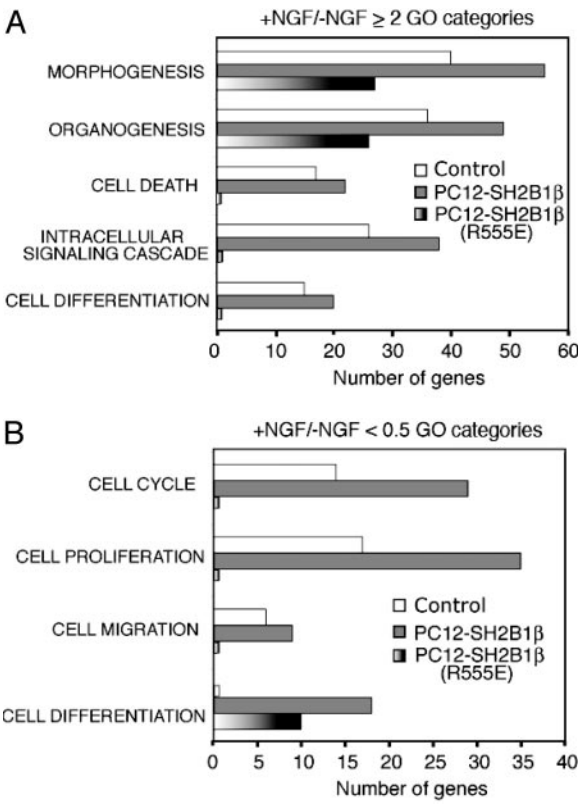


Fig. 2. Functional Classification of NGF-Responsive Genes in Control PC12, PC12-SH2B1β, and PC12-SH2B1β(R555E) Cells

Genes that were either up-regulated to at least twice the basal value by NGF (A) or down-regulated more than 50% by NGF (B) in control PC12, PC12-SH2B1β, and PC12-SH2B1β(R555E) cells were grouped into gene ontology categories. Overrepresented categories with $P < 0.05$ are shown.

that for neuronal differentiation to occur, reduction of gene expression is as important as enhancement of gene expression. Twelve known genes exhibited more than a 50% decrease in expression in response to NGF in control cells and a further 50% or greater decrease in NGF responsiveness in PC12-SH2B1β cells (Table 4). Consistent with SH2B1β enhancing NGF-induced neuronal differentiation, a process associated with decreased cellular proliferation, this group of down-regulated genes includes two that encode for proteins involved in DNA replication: Pola2 (polymerase α-subunit II) and Pold1 (DNA polymerase δ, catalytic subunit). Polymerase α-subunit is required for DNA replication, whereas DNA polymerase δ possesses 3′ exonuclease activity that is important for repair during DNA replication (37). Expression of SH2B1β(R555E) dampened NGF-induced inhibition of expression of only one EST. Dampening of NGF-induced expression of no known genes (supplemental Table S4) achieved our predetermined criterion of at least 50%.

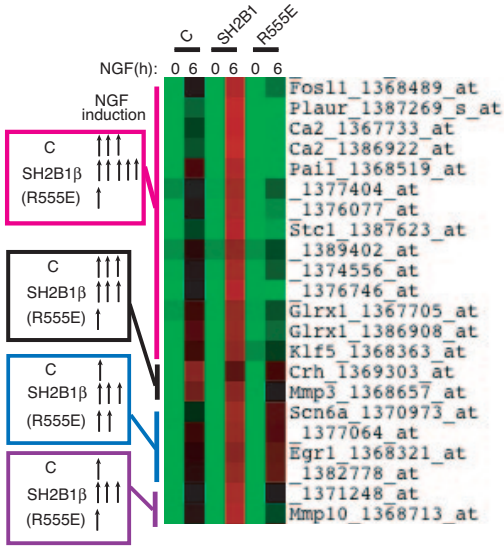
SH2B1β Increases the Expression of a Subset of NGF-Responsive Genes

To systematically identify genes whose expression was substantially increased in response to NGF and whose NGF-induced expression was further substantially increased by overexpression of SH2B1β and/or substantially dampened by overexpression of SH2B1β(R555E), we applied a hierarchical clustering algorithm based on Pearson correlation coefficients to group genes based on the similarity of NGF-dependent expression patterns among the three stable cell lines (Fig. 3). *Red* represents increased mRNA levels, *green* denotes decreased mRNA levels, and color intensity represents the magnitude of the expression ratio. This analysis revealed, in addition to Plaur and Glrx1, which met our most stringent criteria of opposing 2-fold or greater differences in NGF-regulated gene expression in PC12-SH2B1β and PC12-SH2B1β(R555E) cells *vs.* control PC12 cells, several genes whose regulation followed the same pattern but did not quite meet the criteria of at least 2-fold differences (in opposing directions) for both PC12-SH2B1β and PC12-SH2B1β(R555E) cells. NGF-regulated (known) genes that fit both of our criteria [greatly increased NGF induction in PC12-SH2B1β cells and significantly reduced NGF induction in PC12-SH2B1β(R555E) cells] include Fosl1 (encoding Fos-like antigen 1), Plaur (uPAR), Ca2 (carbonic anhydrase 2), Pai1 (serine or cysteine proteinase inhibitor, member 1), Stc1 (stanniolcalcin 1), Glrx1 (glutaredoxin 1), and Klf5 (kruppel-like factor 5). NGF-sensitive genes fitting just one of the criteria [increased NGF responsiveness in PC12-SH2B1β cells or decreased responsiveness in PC12-SH2B1β(R555E) cells compared with control cells] included Mmp10 (increased NGF responsiveness in PC12-SH2B1β cells) and Mmp3 and Crh (CRH) (decreased responsiveness in PC12-SH2B1β(R555E) cells). The NGF-induced expression (+NGF/−NGF) for genes representing each of these categories is listed in Fig. 3B. NGF-induced expression of Scn6a (sodium channel, voltage-gated, type 6, α-polypeptide) and Egr1 (early gene response 1) was also greatly enhanced in PC12-SH2B1β cells compared with control cells, but these genes were not explored further because their NGF-induced expression was also enhanced, albeit to a lesser extent, in PC12-SH2B1β(R555E) cells.

QT-PCR Confirms SH2B1 Enhancement of Expression of NGF-Regulated Genes Identified Using Microarray Analysis

To confirm NGF and SH2B1β regulation of expression of genes identified from the Affymetrix gene array analysis, we performed QT-PCR on representative genes from among the above genes showing by hierarchical clustering algorithm significantly increased (four to seven times greater) NGF-induced expression in PC12-SH2B1β cells *vs.* control cells (Mmp10), substantially decreased (by 33–68%) NGF-induced expression in PC12-SH2B1β(R555E) cells *vs.* control cells (Mmp3), or both (Plaur, Ca2, Stc1, and Glrx1) (Fig. 3C). In general, the levels of NGF-induced gene

(A)



(B)

	C +NGF/-NGF	SH2B1β +NGF/-NGF	R555E +NGF/-NGF
Plaur	9.78	56.16	4.44
Ca2	17.62	92.12	8.41
Ca2	12.00	60.29	7.39
Stc1	3.12	20.87	2.05
Glrx1	6.10	22.88	1.83
Glrx1	3.77	12.75	1.97
Mmp3	114.80	119.00	37.1
Mmp10	38.02	271.78	45.24

(C)

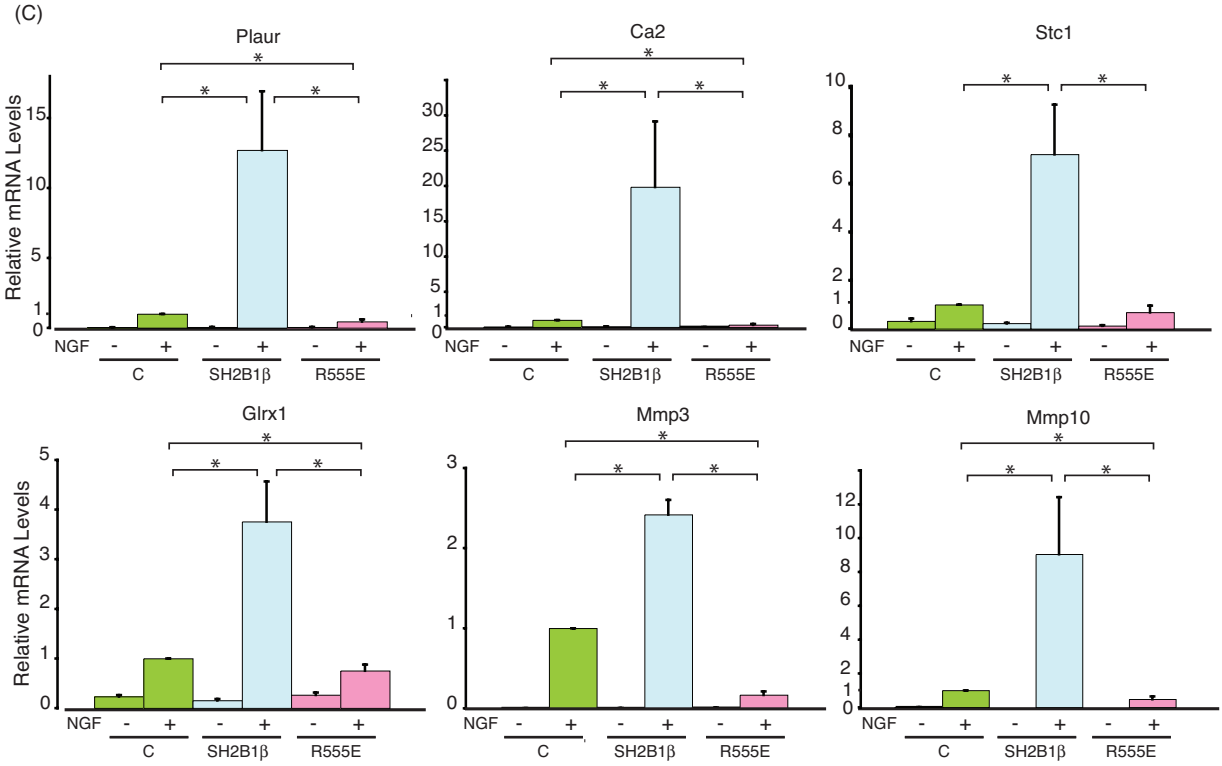


Fig. 3. NGF-Responsive Genes that Are Highly Regulated by SH2B1β

A, NGF-induced gene expression profiles in control PC12 (C), PC12-SH2B1β (SH2B1β), and PC12-SH2B1β(R555E) (R555E) cells were clustered and visualized using Eisen software (http://rana.lbl.gov/EisenSoftware.htm). Four different clusters of genes that show NGF-induced gene expression in all three cell types are shown. For each gene, *black* represents the average value of all six probe sets. The relative intensity of *red* represents the degree of increased mRNA levels, and the relative intensity of *green* represents the degree of decreased mRNA levels. The number of *arrows* represents the relative levels of increase. B, Microarray data for selected genes from each of the four clusters. C, Gene expression levels for six of the genes shown in A were verified by QT-PCR, normalized first to levels of GAPDH gene expression and then to levels of gene expression seen in control PC12 cells treated with NGF. Means ± SEM from three to four experiments are shown. *, Statistically significant differences ($P < 0.05$) using a one-tailed, paired Student's *t* test.

expression in PC12-SH2B1β cells or PC12-SH2B1β(R555E) cells relative to control cells determined by QT-PCR paralleled those found by microarray analysis. Thus, the expression of all six genes was substantially increased in response to NGF in control PC12 cells. Also as predicted, compared with control cells, the NGF-stimulated response was substantially greater in PC12-SH2B1β cells for Plaur, Ca2, Stc1, Glrx1, and Mmp10 and lower in PC12-SH2B1β(R555E) cells for Plaur, Ca2, Glrx1, and Mmp3. In addition, NGF-induced expression was modestly greater in PC12-SH2B1β cells than in control cells for Mmp3 and modestly lower in PC12-SH2B1β(R555E) cells *vs.* control cells for Mmp10. Thus, all six of the genes showed an increased NGF-induced expression when SH2B1β was overexpressed, and five of the six genes showed decreased NGF-induced expression when the dominant-negative SH2B1β(R555E) was expressed, consistent with these genes being regulated by SH2B1β and mediating at least in part SH2B1's effect on NGF-induced neuronal differentiation.

Time Course of NGF-Induced Expression of SH2B1β-Regulated Genes

To determine whether overexpression of SH2B1β increases overall NGF-induced expression of genes or simply shifts the time course of NGF-induced changes in expression, we used QT-PCR to compare the time course of NGF inducement of expression of Stc1, Plaur, Mmp3, Mmp10, Ca2, and Glrx1 genes in control and PC12-SH2B1β cells (Fig. 4). For all genes tested, the time of onset of NGF-induced gene expression appeared similar in control and PC12-SH2B1β cells. Similarly, expression of all tested genes was elevated at multiple time points in PC12-SH2B1β compared with control cells, and the majority of the tested genes had their highest level of induction at 4 or 6 h in both control and PC12-SH2B1β cells. Thus, for no gene could the difference in level of NGF induction in PC12-SH2B1β cells compared with control cells seen after 6 h of NGF be attributed to a different time course of NGF responsiveness. However, it is interesting to note that NGF-induced expression of both Mmp3 and Mmp10 was prolonged in PC12-SH2B1β cells compared with control cells. In contrast to control cells in which Mmp3 and Mmp10 expression declined to near basal values after 24 h of NGF, Mmp3 and Mmp10 expression in PC12-SH2B1β cells remained elevated (Mmp3) or may have even been still rising (Mmp10) after 24 h. These data indicate that SH2B1β both enhances and prolongs NGF induction of expression of a subset of NGF-sensitive genes.

Effect of SH2B1β on NGF-Induced uPAR Protein Level and uPAR Subcellular Distribution

Strikingly, proteins encoded by three of the genes showing the greatest induction by NGF at 6 h as well as the greatest enhancement of that induction by SH2B1β or diminishment of that induction by SH2B1β(R555E) (uPAR, MMP3, and MMP10) fall in the same protease cascade (Fig. 5). In this cascade, uPAR binds the enzymatically inactive proform of urokinase plasminogen activator (pro-uPA), allowing it to be cleaved by cathepsins and thereby activated. The activated uPA proteinase subsequently cleaves inactive plasminogen to form enzymatically active plasmin, which in turn cleaves inactive pro-MMPs to form enzymatically active MMPs. This cascade has been implicated in neurite outgrowth and more generally in cell differentiation, tissue remodeling, cell invasiveness, and wound healing (reviewed in Refs. 38 and 39). Because of the high level of both NGF and SH2B1β regulation of gene expression for uPAR, MMP3, and MMP10, the identification of Plaur as a primary response gene, and the previously documented critical role of uPAR in NGF-induced neuronal differentiation, we studied further the effect of SH2B1β on uPAR, MMP3, and MMP10. To confirm that the changes in NGF-induced Plaur gene expression levels seen in PC12-SH2B1β and PC12-SH2B1β(R555E) cells results in corresponding changes in the levels of uPAR protein, we examined levels of uPAR protein in control, PC12-SH2B1β, and PC12-SH2B1β(R555E) cells by both Western blot and immunocytochemistry. In Western blots of lysates from control cells, 4 h of NGF treatment transiently increased levels of uPAR protein (Fig. 6A). In PC12-SH2B1β cells, NGF increased levels of uPAR protein at both 4 and 6 h, with the 6-h levels being significantly greater than in control cells. Compared with control cells, PC12-SH2B1β(R555E) cells showed reduced levels of both basal and NGF-induced uPAR protein at all time points. Levels of expression of SH2B1β and SH2B1β(R555E) were similar to each other and all three cell lines expressed similar levels of actin, indicating that the differences seen in uPAR levels were not due to differences in the amount of protein loaded. The reason the magnitude of the uPAR signal in PC12-SH2B1 cells relative to control PC12 cells was lower than what the gene expression studies would predict is not known. However, the fact that the difference in levels in NGF-induced protein expression between control and SH2B1β PC12 cells is greatest at 6 h raises the possibility that even greater differences would be detectable at later times.

To confirm the relative levels of NGF-induced uPAR expression and to determine whether SH2B1β affects the spatial distribution of uPAR, we immunostained control PC12, PC12-SH2B1β, and PC12-SH2B1β(R555E) cells for uPAR after 0, 7, or 24 h of 100 ng/ml NGF. Immunostaining was performed under nonpermeabilizing conditions to allow better visualization of uPAR associated with the plasma membrane. In all three cell lines, uPAR was observed at the cell surface in adhesion complex-like puncta (Fig. 6, G, I, K, M, and O), consistent with uPAR

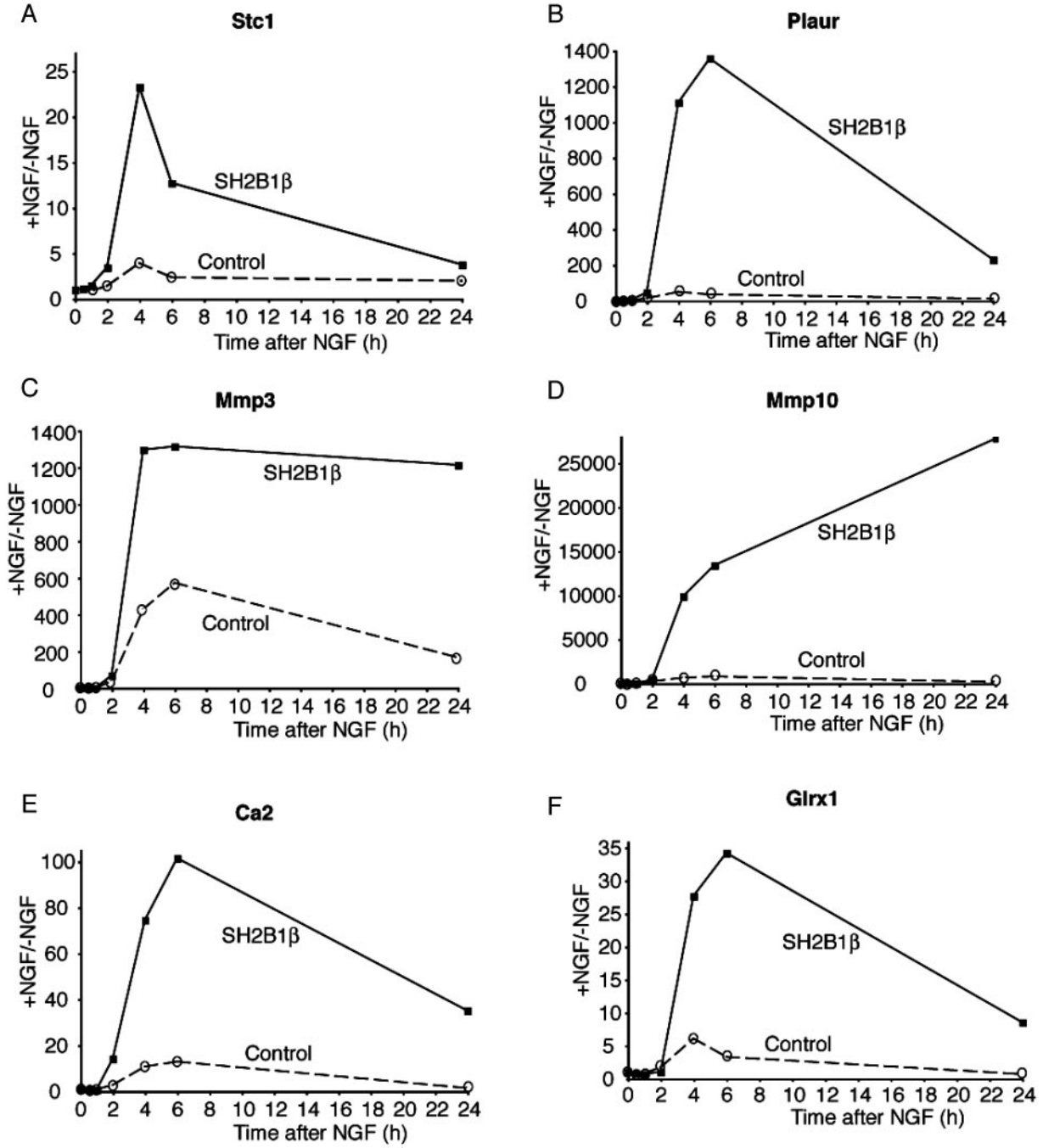


Fig. 4. Time Course of SH2B1β Enhancement of Expression of Representative NGF-Regulated Genes

Control PC12 or PC12-SH2B1β cells were treated with 100 ng/ml NGF for 0 or 30 min or 1, 2, 4, 6, or 24 h. Relative gene expression levels were determined using QT-PCR and normalized to levels of GAPDH gene expression.

being a glycosylphosphatidylinositol-linked protein and its known interaction with proteins (*e.g.* integrins) in adhesion complexes (reviewed in Ref. 40). In all three cell lines, plasma membrane uPAR was present throughout the cell body of the neurite and at growth cones (Fig. 6, G, I, K, and M, and a darker exposure of Fig. 6O, data not shown). NGF treatment increased levels of uPAR at the plasma membrane of PC12-SH2B1β cells at both 7 and 24 h (compare Fig. 6G and 6I to 6E) as well as in control and SH2B1β(R555E) cells (data not shown). At 24 h after NGF addition, plasma membrane uPAR immunostaining was clearly greater in PC12-SH2B1β cells (Fig. 6L) and lower in PC12-SH2B1β(R555E) cells (Fig. 6O) than in control PC12 cells (Fig. 6K). The previously reported requirement of uPAR for NGF-induced neurite outgrowth (33) combined with the ability of SH2B1 to greatly enhance both expression of Plaur gene and plasma membrane levels of uPAR protein reported here suggest that SH2B1 enhances NGF-induced

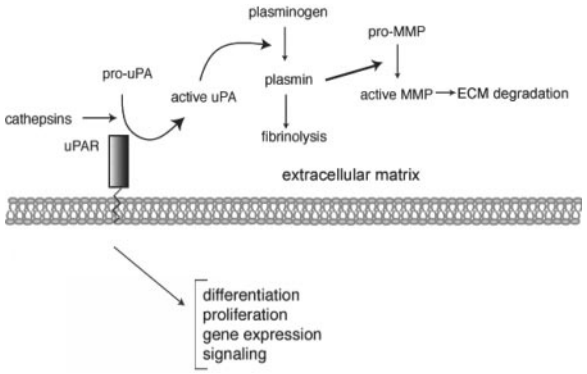


Fig. 5. Physiological Functions of uPAR/MMP Activation Cascade

Cell surface-localized uPAR, together with cathepsins, binds to pro-uPA and converts inactive pro-uPA to active uPA. The active uPA converts plasminogen to plasmin, and the active plasmin cleaves and thereby activates MMPs. Active MMP has been shown to regulate ECM degradation.

neurite outgrowth at least in part by mediating or enhancing the NGF-induced expression of Plaur.

NGF-Induced MMP3/10 Activity Is Elevated in PC12-SH2B1β Cells and Suppressed in PC12- SH2B1β(R555E) Cells

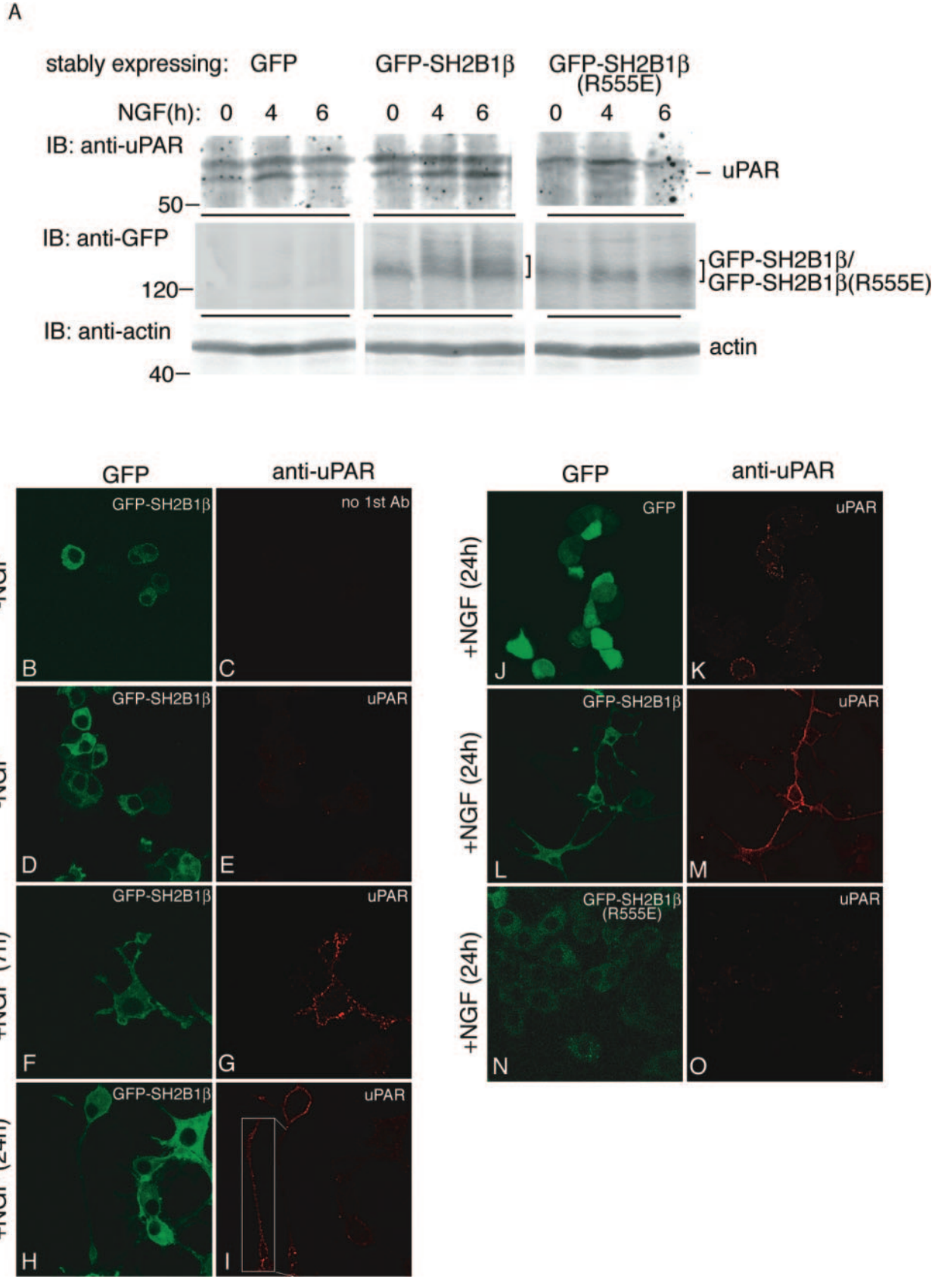
We next examined the effect of NGF and SH2B1β on the protease activity of MMP3 and MMP10. Because of their similar size and substrate specificity and in the absence of immunoprecipitating antibodies that could distinguish between MMP3 and MMP10, we assayed MMP3 and MMP10 together. Conditioned medium was collected from cells treated with 100 ng/ml NGF for various times, concentrated, and assayed for the presence of MMP3 and MMP10 using a zymogram gel containing casein as substrate. When purified human MMP3 was used as a positive control, as expected, a cleared band was observed at M_r approximately 50,000 (nonreducing condition), consistent with MMP3 degrading the casein (Fig. 7A). Rat MMP3 migrates as a 60-, 62-kDa doublet under reducing conditions. It was therefore expected to migrate slightly slower than human MMP3 (41). MMP3 and MMP10 are approximately the same size and cannot be differentiated in this assay. By 6 h of NGF treatment, control cells showed noticeable NGF-induced casein-degrading activity at a molecular weight consistent for MMP3/10. This protease activity continued to increase with increasing incubation times (8 and 10 h) with NGF. Consistent with SH2B1β increasing NGF-induced levels of Mmp3/Mmp10 gene expression, this NGF-dependent protease activity was greater in magnitude in NGF-treated PC12-SH2B1β cells and substantially decreased in magnitude in NGF-treated PC12-SH2B1β(R555E) cells compared with control cells.

SH2B1β Enhances NGF-Induced Neurite Outgrowth of PC12 Cells through Matrigel

Like uPAR activity, MMP3 activity has been associated with neurite outgrowth. However, unlike uPAR, its down-regulation (by use of antisense RNA) did not affect the number or length of neurites produced by PC12 cells on a two-dimensional tissue culture surface. Rather, down-regulation of MMP3 blocked the ability of neurites to penetrate through a Matrigel barrier (36). Combined with the finding that SH2B1β enhances the activity of MMP3/10, this latter finding suggests that SH2B1β should enhance growth cone migration and invasion through an ECM barrier. To test this, control PC12 cells and PC12-SH2B1β cells were plated onto the *cis*-plane of a transwell membrane overlaid with growth factor-reduced Matrigel (Fig. 8A). NGF (100 ng/ml) was added every other day to the outer chamber for 4 d. Cells were then fixed and immunostained for neuronal β-tubulin. Neurites that traversed to the *trans*-plane of the membrane were counted. The number of neurites that invaded through the Matrigel to the *trans*-plane in cells expressing GFP-SH2B1β was found to be over twice (230%) the number of invading neurites from control cells (Fig. 8B). The neurons themselves were found not to traverse the *trans*-plane of the membrane.

Inhibition of SH2B1 Suppresses NGF-Induced Expression of Plaur, Mmp3, and Mmp10

The ability of overexpression of wild-type SH2B1β to enhance and the dominant-negative SH2B1β(R555E) to inhibit NGF-induced expression of Plaur, Mmp3, and Mmp10 indicates that endogenous SH2B1β regulates the NGF induction of these three genes. To test this more directly, we used an RNA interference (RNAi) approach to stably reduce levels of endogenous SH2B1 in PC12 cells. As shown in Fig. 9A, the level of endogenous SH2B1 in cells stably expressing a 21-nucleotide long shRNA duplex targeted against SH2B1 (PC12-shSH2B1 cells) was reduced to less than 50% the level in cells stably expressing the vector only (shControl cells) when normalized to levels of β-tubulin. As predicted from the experiments using PC12-SH2B1β and PC12-SH2B1β(R555E) cells, PC12-shSH2B1 cells exhibited substantially reduced NGF-induced neurite outgrowth compared with control cells (Maures, T., L. Chen, and C. Carter-Su, manuscript in preparation). In support of endogenous SH2B1 enhancing NGF-induced expression of Plaur, Mmp3, and Mmp10, PC12-shSH2B1 cells exhibited substantially suppressed NGF-induced levels of expression of Plaur (by 56%), Mmp3 (by 47%), and Mmp10 (by 76%) compared with control cells (Fig. 9B), levels of reduction consistent with the level of reduction in SH2B1β protein. Levels of GAPDH gene expression were not affected by NGF, nor were they significantly different for the two cell lines, indicating that the differences in NGF induction seen in Plaur,



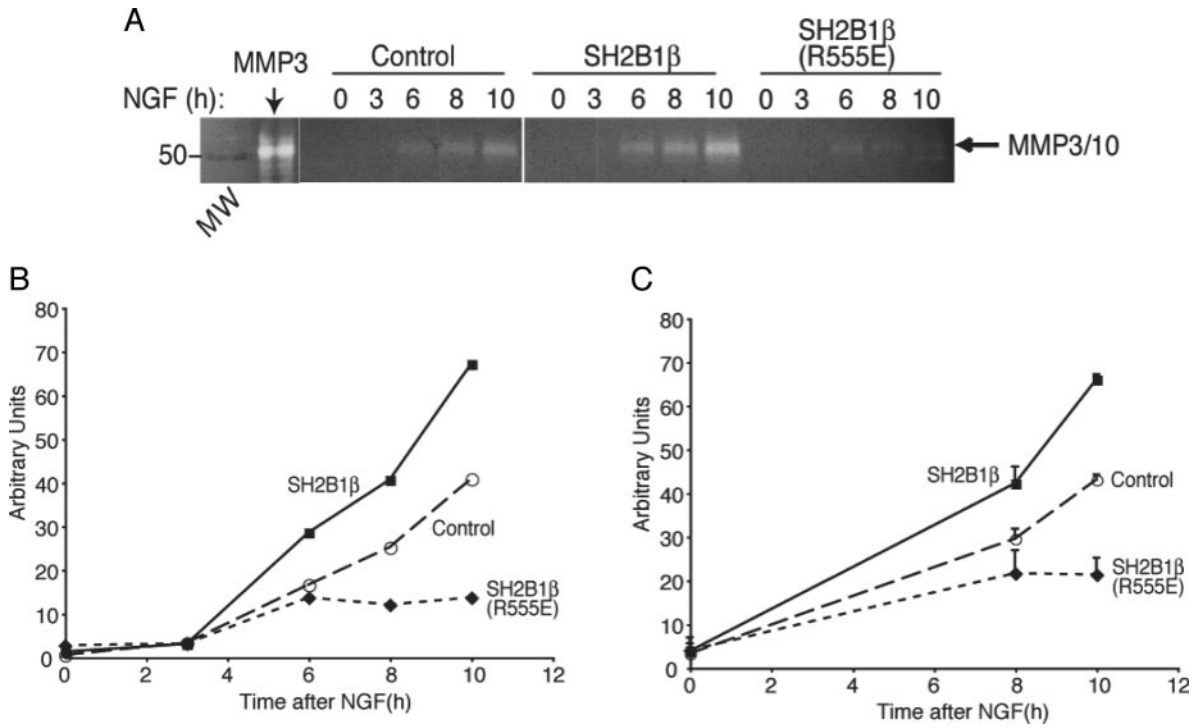


Fig. 7. SH2B1β Enhances NGF-Induced MMP3/MMP10 Activity

A, Control PC12, PC12-SH2B1β, or PC12-SH2B1β(R555E) cells were treated with 100 ng/ml NGF for 0, 3, 6, 8, and 10 h. Proteins in concentrated conditioned medium were separated in casein-containing, prestained zymogram gels and then developed for 16 h. A representative gel is shown. B, The MMP3/10 activity in A was quantified by band intensity using NIH Image software. C, NGF-induced MMP3/10 activity was quantified for three independent experiments. Means ± SEM are shown. MMP3/10 activity levels of the three cell lines are significantly different ($P < 0.05$, one-tailed, paired Student's *t* test) at 10 h, and by 8 h, MMP3/10 activity in PC12-SH2B1β cells was elevated above control cells ($P > 0.05$).

Mmp3, and Mmp10 expression in the different cell lines were not due to nonspecific effects of the shRNAi on gene transcription or cell viability.

DISCUSSION

Microarray Analysis Identifies Novel NGF-Responsive Genes Likely to Be Important for NGF-Induced Neuronal Differentiation

In this study, we applied genome-scale gene expression analysis to identify SH2B1β-regulated genes and to gain insight into whether the adaptor protein SH2B1β enhances NGF-induced regulation of all NGF-sensitive genes, as predicted if SH2B1 simply potentiates NGF activation of TrkA, or whether it alters expression of a subset of NGF-sensitive genes as predicted if SH2B1 were acting downstream of TrkA. Because we were interested in the early events of NGF-induced neuronal differentiation, including neurite outgrowth, we looked at the gene expression profiles of the well-defined sympathetic neuron model PC12 cells before and after 6 h treatment of NGF.

Although our main goal was to identify genes that are regulated by SH2B1β, our microarray analysis also

Fig. 6. SH2B1β Enhances NGF-Induced uPAR Protein Expression but Does Not Affect the Subcellular Distribution of Cell Surface-Localized uPAR

A, PC12 cells stably expressing GFP, GFP-SH2B1β, or GFP-SH2B1β(R555E) were incubated with 100 ng/ml NGF for 0, 4, and 6 h. Proteins in a whole-cell lysate were separated by SDS-PAGE under nonreducing conditions, and uPAR protein levels were determined by immunoblotting with antibody to rat uPAR. Expression levels of GFP-SH2B1β, GFP-SH2B1β(R555E), and actin were determined under reducing conditions by immunoblotting proteins in whole-cell lysates with α-GFP or α-actin, respectively. The molecular weight markers are noted on the *left* (n = 2). B, PC12 cells stably expressing GFP, GFP-SH2B1β, or GFP-SH2B1β(R555E) plated on Matrigel-coated coverslips were incubated with 100 ng/ml NGF for 0, 7, or 24 h as indicated and fixed with 4% paraformaldehyde but not permeabilized before addition of monoclonal uPAR antibody and antimouse Alexa Fluor 555. Representative confocal images are shown. The *inset* in I is an image of a different focal plane that focused more on the neurite. The experiment was repeated three times with similar results.

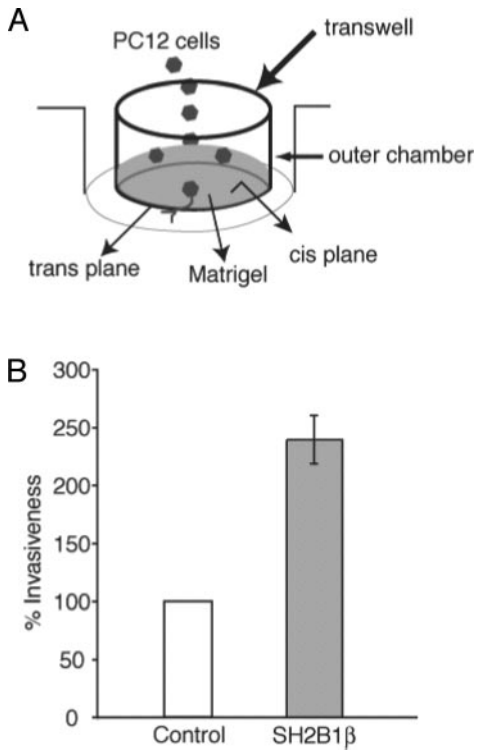


Fig. 8. SH2B1β Enhances NGF-Induced Neurite Invasiveness of PC12 Cells

A, Schematic of transwell system used to measure neurite invasiveness. Cells were added onto growth factor-reduced Matrigel on the *cis*-plane of a transwell insert. Neurite invasiveness was assessed by counting the neurites appearing on the *trans*-plane of the transwell insert. B, Control PC12 cells or PC12-SH2B1β cells were added to the Matrigel on the *cis*-plane of a transwell insert, and 100 ng/ml NGF was added to the outer chamber. After 4 d, the cells on the insert were fixed and immunostained with anti-β-tubulin and Alexa Fluor 555. Neurites that traversed to the *trans*-plane were counted. Means ± SEM are shown for n = 3.

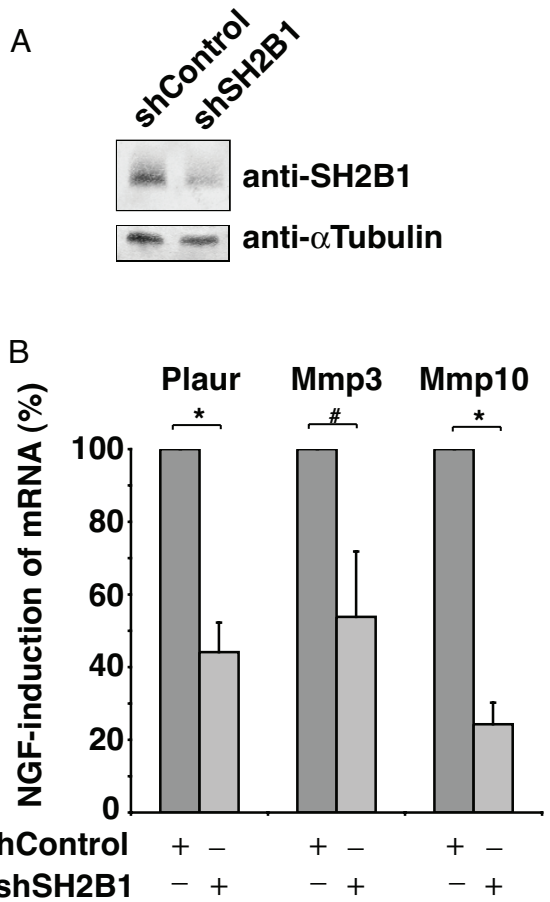


Fig. 9. The siRNA-Mediated Knockdown of Endogenous SH2B1β Inhibits NGF-Dependent Induction of Plaur, Mmp3, and Mmp10

A, Proteins in lysates of PC12 cells stably expressing the control shRNA (shControl) or shRNA targeted against SH2B1 (shSH2B1) were separated by SDS-PAGE and immunoblotted with α-SH2B1 and then anti-α-tubulin as a loading control. B, After incubation in serum-free medium overnight, PC12 cells expressing control shRNA or shRNA targeted against SH2B1 were incubated with or without 50 ng/ml NGF for 6 h. The NGF-dependent induction of mRNA for Plaur, Mmp3, and Mmp10 was quantified via QT-PCR. Gene expression in the presence of NGF was divided by expression in the absence of NGF and then normalized to levels of GAPDH expression in the presence of NGF divided by expression in the absence of NGF. Results were then further normalized to those obtained using shControl cells. Means ± SEM are shown for n = 3. *, $P < 0.05$ using a one-tailed, paired Student's *t* test; #, $P = 0.06$.

identified over 1000 genes whose expression was substantially regulated by NGF, many more than identified previously. Several other studies have reported NGF-induced gene expression on a large scale (42–49); however, most of these studies focused on the late response genes induced by 4 or more days of NGF treatment. The few studies looking at earlier time points identified a limited number of NGF-sensitive genes, the majority of which were also identified as NGF-sensitive genes in our microarray analysis. For example, Chou *et al.* (50) used gene expression profiling of PC12 cells at 1, 6, and 24 h after NGF treatment to identify nine genes of 588 genes and ESTs that were highly regulated (>300 or <33% of control) in response to NGF. Expression of the two genes [Mmp10 and Vgf encoding VGF nerve growth factor inducible/VGF8a protein/neurosecretory protein VGF precursor] found to be highly up-regulated after 6 h treatment with NGF were also highly up-regulated (7- and 38-fold, respectively) by 6 h of NGF in our microarray study. Among the NGF-regulated genes identified by Brown *et al.* (49) using targeted display to identify NGF-sensitive genes after 2, 4, and 6 h, our microarray data showed similar up-regulation by NGF of Catna1 encoding α-catenin, Odc1 encoding ornithine decarboxylase, Gsr encoding glutathione reductase, and Nup54 encoding p54 nucleoporin and down-regulation of Gucy1b3 encoding β-subunit of soluble guanylate cyclase and Chaf1A encoding p150 chromatic assembly factor. Finally, our microarray also identified as highly NGF dependent six genes identified by Herschman's group (33,

48, 51) as being induced by 1–4 h of NGF but not epidermal growth factor (EGF) treatment using representational difference analysis: Arc (encoding activity-regulated cytoskeletal protein), Mmp13 (encoding MMP13/collagenase-3), Serpine1 (encoding plasminogen activator inhibitor-1 precursor/PAI-1), VH6/MKP-3/Dusp6 (encoding dual specificity phosphatase 6/MAPK phosphatase 3), Plaur, and Nid67 (encoding NGF-induced differentiation clone 67 protein).

In addition to these previously identified NGF-induced genes, our microarray data identified close to 500 additional genes and ESTs whose expression was at least doubled with 6 h of NGF treatment and close to 150 additional genes and ESTs whose expression was at least tripled. More than 500 genes and ESTs showed an NGF-induced decrease in expression of 50% or greater, with over 100 showing a 66% or greater decrease. The NGF-sensitive genes identified in our study that were up-regulated at least 2-fold were overrepresented in the gene ontology categories of morphogenesis, organogenesis, cell death, intracellular signaling, and cell differentiation, consistent with NGF promoting differentiation and survival of sensory and sympathetic neurons. Those genes that were down-regulated at least 50% by NGF were overrepresented in the categories of cell cycle, proliferation, and migration, consistent with the known decrease in cell proliferation and movement that occurs during differentiation. Some of these NGF-regulated genes [*e.g.* genes encoding integrin α1, lectin, galactose binding, soluble 3 (45), bradykinin B2 receptor (46), and Bip3 (52)] had been shown previously to be regulated by NGF in PC12 cells using a variety of techniques, although not always at such an early time point (6 h) after NGF addition. Other genes (*e.g.* Glrx1 encoding glutaredoxin 1, Ca2 encoding carbonic anhydrase-II, and Stc1 encoding stanniolcalcin 1) were revealed for the first time to be highly responsive to NGF. Many of the proteins encoded by our newly identified NGF-regulated genes, including Glrx1, Stc1, and Ca2, had, however, been previously identified in neurons and, in some cases, been implicated in neuronal differentiation and/or function. For instance, glutaredoxin 1 is thought to play an important role in maintaining nerve cell function in the presence of oxidative stress, at least in part by catalyzing the removal of glutathione from S-glutathionylated proteins (reviewed in Ref. 53). Because even small changes in the redox state of a cell have been shown to switch cells from proliferation to differentiation or vice versa (reviewed in Ref. 54), our finding that NGF greatly up-regulates glutaredoxin 1 expression raises the possibility that glutaredoxin 1 also plays an important role in NGF-induced neuronal differentiation. Proteins that regulate cellular pH, such as carbonic anhydrase II, are also thought to be especially important in the nervous system because electrical activity can elicit rapid changes in cellular pH (55). Our finding that carbonic anhydrase II, found in certain subsets of nerves, is rapidly and profoundly up-regulated by NGF in differentiating PC12 cells, and the finding of Dickens *et al.* (56) that tips of extending neurites appear to be more alkaline (by 0.2–0.3 pH units) than the cell body raise the possibility that carbonic anhydrase II plays a specific role in neurite outgrowth. Although not previously reported to be induced by NGF, stanniolcalcin-1, a glycoprotein hormone implicated in the regulation of calcium and phosphate homeostasis, has been reported to be induced during differentiation of a variety of cell types, including human neural crest-derived Paju cells (stimulated by phorbol esters) (57) and murine Neuro-2A neuroblastoma cells (stimulated by dibutyryl cAMP) (58). Decreasing its expression in Neuro-2A cells using antisense oligodeoxynucleotides reduces in neurite outgrowths the level of varicoses, a phenotypic marker of axon formation. This finding led to the suggestion that stanniolcalcin-1, which is found in neurons in human and adult mouse brain, is involved in axonal formation. Clearly, knowledge and further exploration of these proteins and the proteins encoded by the large number of other genes newly found to be NGF sensitive should greatly facilitate our understanding of how NGF induces neuronal differentiation and promotes neuronal survival.

SH2B1β Enhances the NGF Regulation of a Subset of Genes Likely to Be Involved in Neuronal Differentiation

Comparison of the gene expression profiles of control cells and cells stably expressing SH2B1β or the dominant-negative SH2B1β(R555E) revealed a number of NGF-induced and -depressed genes that are differentially regulated by SH2B1β and SH2B1β(R555E). Some genes whose expression is substantially up-regulated by SH2B1β have been reported previously to be expressed in neurons but were not known to be regulated by NGF [*i.e.* Stc1, Glrx1(a), and Scn6a] (59–61). Other NGF-sensitive genes whose NGF stimulation is increased by SH2B1β (*e.g.* Arc, Fosl1/Fra1, Dusp6, Egr1, and Plaur) have been shown previously to be regulated by NGF in neuronal cells (33, 48, 62). The gene products of a few (*i.e.* Plaur and Gap-43/Basp2) have even been implicated previously in NGF-induced neurite outgrowth (33, 63). For the six genes tested (Glrx1, Ca2, Stc1, Mmp3, Mmp10, and Plaur), QT-PCR analysis confirmed both their up-regulation by NGF and the ability of SH2B1β to increase the magnitude of that up-regulation. QT-PCR analysis further revealed that for all six of the genes tested, SH2B1β significantly enhanced levels of expression at multiple time points after NGF addition, indicating that the SH2B1β-stimulated levels of expression seen at 6 h of NGF treatment in the microarray were not simply a consequence of a shift in the time course of NGF activation. Western blotting, activity assays, and/or immunocytochemistry indicated that increased expression of Plaur, Mmp3, and Mmp10 genes in PC12-SH2B1β cells results in increased protein expression of uPAR and MMP3/10 and that decreased gene expression of Plaur, Mmp3, and Mmp10 genes in PC12-SH2B1B(R555E) cells results in decreased protein expression of uPAR and MMP3/10.

It is important to note, however, that although the NGF responsiveness of a number of NGF-sensitive genes was enhanced by overexpressing SH2B1, there were similar numbers of NGF-sensitive genes whose expression was not substantially altered by overexpressing SH2B1β. Genes in this latter category included some genes whose expression is considered critical for NGF-induced neuronal differentiation. For example, NGF-induced expression of Anxa2 encoding annexin II/calpactin 1 heavy chain is thought to be critical for NGF-induced neuronal differentiation of PC-12 cells. Polyclonal and monoclonal antibodies directed against annexin II and overexpression of antisense annexin II mRNA inhibit NGF-induced PC12 neurite outgrowth on a plate and within a three-dimensional matrix (64). Annexin II is reported to be a coreceptor for plasminogen and tissue plasminogen activator, which promotes and localizes plasmin generation near the cell surface (65, 66). Although in our microarray, NGF was observed to stimulate the expression of Anxa2 to three times the level seen in the absence of NGF, NGF induction of expression was similar in control, PC12-SH2B1β, and PC12-SH2B1β(R555E) cells. Like Plaur, Vgf, encoding VGF nerve growth factor-inducible protein, is a primary response gene that is specific for NGF and not EGF and peaks in expression in PC12 cells after 2–8 h of NGF (33). Ret encoding the common receptor for glial cell line-derived neurotrophic factor (GDNF) family ligands is thought to be required for GDNF to instruct a subset of $TrkA^{+}$ sensory neurons to adopt a nonpeptidergic sensory neuron fate (67). Although in control cells we observed NGF to substantially increase expression of both Vgf and Ret (by ~7-fold), the NGF-induced expression of these two genes was not altered in PC12-SH2B1β or PC12-SH2B1β(R555E) cells. Taken together, these findings suggest that SH2B1β enhances the expression of a critical subset of NGF-responsive genes implicated in neuronal differentiation.

SH2B1β Enhances the Ability of Nerves to Express uPAR and MMP3/10, Which in Turn Enhances the Ability of Neurites to Invade the Extracellular Matrix

When we looked at the top five genes whose NGF-induced expression at 6 h by microarray analysis was most highly up-regulated in PC12-SH2B1β cells or down-regulated in PC12-SH2B1β(R555E) cells compared with control cells, we were impressed by the fact that three of the protein products (uPAR, MMP3, and MMP10) are involved in the same pathway of ECM degradation, a pathway critical for neurite outgrowth. What was even more striking is that the genes encoding these three proteins were also among the top 12 genes up-regulated by NGF at 6 h in control cells. One of these genes (Plaur) is a known early-response gene regulated by NGF (which promotes neuronal differentiation of PC12 cells) but not EGF (which does not promote neuronal differentiation of PC12 cells), shown previously to be essential for NGF-induced neurite outgrowth and biochemical differentiation (33, 34). These combined observations caused us to focus on uPAR, MMP3, and MMP10 as key mediators of the stimulatory effect of SH2B1 on NGF-induced neurite outgrowth.

The key features of NGF-dependent neuronal growth, such as the initiation and extension of the axon, are contingent upon the formation and motility of the growth cones. uPAR and MMP3 and -10 function together in a pathway to modulate the cell's degradation of the surrounding ECM, allowing the axon to invade or penetrate the ECM. uPAR is a glycosylphosphatidylinositol-linked protein that binds to and activates urokinase plasminogen activator (uPA) at the cell surface. uPAR has been implicated as being vital for NGF-induced differentiation of PC12 cells. Thus, in PC12 cells, anti-uPAR antibody to the extracellular domain of uPAR and antisense RNA targeted to uPAR message both block NGF-induced neurite outgrowth and appearance of biochemical markers of neuronal differentiation (34). uPAR is also thought to be an essential player for neuronal differentiation *in vivo*. uPAR is found in primary neurons and has been implicated in the differentiation of primary neurons. Hayden and Seeds (68) report that uPAR mRNA levels greatly increase during differentiation of cultured cells from mouse dorsal root ganglia. The time course of the transient increase (maximum at 30 h) directly correlated with the differentiation of neurons and the formation of a neuritic network in the regenerating cultures. Levels of uPAR mRNA also increase in sensory neurons after sciatic nerve crush in adult mice (69), and the neocortex of $uPAR^{-/-}$ adult mice has fewer GABAergic interneurons during embryonic and postnatal periods (70, 71).

Activated uPA converts plasminogen to plasmin, which subsequently cleaves inactive MMPs into active extracellular proteases. There are currently 28 identified enzymes in the MMP family, each displaying a propensity for the cleavage of specific substrates. MMP3 and -10 are secreted proteinases that are cleaved by plasmin; they are both capable of degrading a broad spectrum of substrates, including collagen I, III, IV, and V and fibronectin (32). In response to signals like NGF, neurons position these ECM proteases to their growth cones. In PC12 cells, the time course of NGF-induced Mmp3 expression is largely coincident with neuronal differentiation (72), and MMP3 has been implicated in the motility of the growth cone (36, 73). Knocking down MMP3 in PC12 cells blocks NGF-induced neurite invasion or extension through Matrigel, suggesting that MMP3 is vital for axon outgrowth through the ECM. In support of a role of MMPs in axonal outgrowth *in vivo*, MMP3 and MMP10 are found in primary neurons. MMPs have also been implicated in penetration of dorsal root ganglion neurites through the ECM (74). Thus, in the most simplistic explanation of how uPAR, MMP3, and MMP10 mediate neurite outgrowth, NGF causes an increased transcription/translation of uPAR and MMPs, bestowing the cell with a greater proteolytic potential that allows the growth cones to cut through the ECM, clearing a pathway for the neurite. The ability of SH2B1β to greatly increase the expression of uPAR and increase and prolong the ex-

pression of both MMP3 and MMP10 suggests that SH2B1β is required for the degradation of the ECM required for axonal outgrowth of NGF-sensitive nerves. This is supported by our finding that both overexpression of the dominant-negative SH2B1β(R555E) and reduction of expression of endogenous SH2B1 using shRNA to SH2B1 substantially decrease the expression of uPAR, MMP3, and MMP10. It is further supported by the finding that cells overexpressing SH2B1β both increased the amount of enzymatically active MMP3/10 over levels associated with control PC12 cells in response to NGF as well as NGF-induced neurite invasion through Matrigel.

However, the contribution of uPAR and MMPs, and thus SH2B1, to neurite outgrowth may extend beyond this simple explanation. The requirement for functional uPAR during NGF-dependent differentiation of PC12 cells is transient and essential only for the initial hours of NGF exposure (33, 34). Furthermore, not only does depletion or inhibition of plasma membrane uPAR block NGF-induced neurite outgrowth in a culture plate, but it also blocks biochemical differentiation assessed as expression of COX-1 and type II sodium channel and expression of both MMP3 (transin) and MMP1 (collegenase I) (33). These observations suggest that uPAR is an immediate-early gene product of NGF whose function may be required for induction of a wave of secondary response genes important for differentiation. Indeed, uPAR has also been reported in other cell types to activate a variety of intracellular signaling pathways and increase levels of a variety of signaling molecules. The pathways and molecules include Janus kinase 1/signal transducer and activator of transcription 1 (75), MEK/ERK (76), the Src family kinase hck (77), and protein kinase Cε (78) pathways as well as diacylglycerol (79, 80), cAMP (81), calcium released from internal stores (82), and inositol phosphate turnover (83). In other cell model systems, uPAR has been reported to form stable complexes with integrins that alter the adhesive properties of the cells (84, 85). Thus, NGF-induced neuronal differentiation and enhancement of that differentiation by SH2B1β may also require uPAR activation of cellular signaling pathways.

In a similar vein, both axons and growth cones appear to require MMPs to not only extend into the ECM but also to remove inhibitor proteins and process guidance cues (reviewed in Ref. 86). MMP3 has been implicated in synaptogenesis (87) and found to confer neuronal survival properties by removing Fas ligand from the neuronal surface, thereby protecting against Fas-induced apoptosis (88). Thus, in intact neurons, as a consequence of promoting and prolonging the NGF-induced expression of Mmp3 and Mmp10, SH2B1β may not only be enhancing neurite invasion of the ECM, it may also be enhancing neuronal survival and affecting synaptogenesis. A specific role for SH2B1 in neuronal survival is consistent with the finding of Qian *et al.* (20) that antibodies to SH2B1α introduced into dissociated sympathetic neurons maintained in the presence of NGF promote cell death and that introduction of a dominant-negative form of SH2B1α into explants of sympathetic ganglia maintained in the presence of NGF result in axonal degeneration.

Cellular Mechanism by which SH2B1β Enhances Expression of uPAR and MMP3

Mmp3 expression has been shown to be dependent upon uPAR expression (33, 48), making it likely that SH2Bβ1 enhancement of MMP3 expression is secondary to SH2B1β enhancement of uPAR expression. The molecular mechanism by which SH2B1β enhances NGF-induced Plaur gene expression is unknown. As discussed previously, one hypothesis is that SH2B1 increases NGF induction of TrkA (29), which would be expected to result in an increase in all cellular responses to NGF, including expression of Plaur. However, our data showing that SH2B1β increases expression of only a subset of NGF-induced genes and previous data showing that NGF induction of TrkA is not enhanced in PC12-SH2B1β(R555E) cells (21, 30) argue against this being the primary explanation for how SH2B1β stimulates expression of Plaur. Recent findings suggest that NGF causes the recruitment of c-Fos and JunB to an AP-1 regulatory element within the uPAR promoter (89), raising the possibility that SH2B1 somehow facilitates that process either directly in its role as an adapter protein or indirectly by promoting expression of c-Fos or JunB.

The work of Qian and colleagues (20, 29) is consistent with SH2B1 enhancing NGF activation of ERKs 1 and 2, which would be expected to increase the expression of c-Fos. Arguing against this hypothesis, however, is that although overexpression of SH2B1β has a modest enhancing effect on NGF activation of phosphorylation of ERKs 1 and 2 (21, 30), neither overexpression of SH2B1β(R555E) (21) nor reduction of endogenous SH2B1 (Maures, T., L. Chen, and C. Carter-Su, manuscript in preparation) inhibits NGF-induced ERK phosphorylation. In support of the conclusion that SH2B1β does not enhance NGF-induced Plaur expression and neuronal differentiation solely by enhancing NGF-induced activation of TrkA or ERKs 1 and 2, GDNF-induced neuronal differentiation of PC12 cells stably overexpressing GDNF receptor α1 and RET is also enhanced by overexpression of SH2B1β but suppressed by overexpression of SH2B1β(R555E) and by reduction of endogenous SH2B1 using shRNA to SH2B1 (90). However, GDNF stimulation of phosphorylation of ERKs 1 and 2 and Akt was not appreciably suppressed by overexpression of SH2B1β(R555E).

Taken together, these findings suggest that SH2B1 enhances both NGF- and GDNF-induced neuronal differentiation at a point downstream of or parallel to ERKs. The additional findings that SH2B1β enhancement of NGF-induced Plaur expression and neurite outgrowth appear to require nuclear cytoplasmic shuttling of SH2Bβ1 (30) (Maures, T., L. Chen, and C. Carter-Su, manuscript in preparation) open up the

possibility of a novel pathway that requires SH2B1 to cycle through the nucleus.

Conclusion

In conclusion, our microarray analysis of PC12 cells treated with or without NGF for 6 h identified about 1000 genes and ESTS whose expression was increased or decreased by NGF by a factor of 2 or more, many of which were not previously recognized as being NGF-responsive genes. NGF-induced regulation of a substantial subset of these genes was enhanced by overexpressing wild-type SH2B1β and/or suppressed by overexpressing dominant-negative SH2B1β(R555E). Another subset of NGF-sensitive genes implicated previously in NGF induction of neuronal differentiation were unaffected by overexpression of SH2B1β or SH2B1β(R555E), suggesting that SH2B1β acts primarily downstream of TrkA. Three of the up-regulated genes (Plaur, Mmp3, and Mmp10) were noted to be particularly sensitive to both NGF and SH2B1β. Their regulation by both NGF and SH2B1β at the gene level was confirmed by QT-PCR and at the protein level by immunoblotting, immunocytochemistry, and/or functional assays. The protein products of these three genes lie in the same functional pathway of ECM degradation critical for neurite outgrowth; uPAR has been shown previously to be critical for NGF-induced neuronal differentiation and MMP3 for NGF-induced neurite growth through a three-dimensional matrix. These observations suggest that uPAR, MMP3, and MMP10 are likely to play a critical role in the ability of SH2B1 to facilitate NGF-induced neuronal differentiation and neurite outgrowth. A critical role is further suggested by our findings that depletion of endogenous SH2B1β suppresses the ability of NGF to enhance expression of all three genes and that overexpression of SH2B1β promotes neurite extension through a three-dimensional matrix. The additional finding that nuclear cytoplasmic shuttling of SH2B1β is required for SH2B1β to promote NGF-dependent neuronal differentiation makes it intriguing to speculate that nuclear SH2B1β directly facilitates expression of a subset (*e.g.* Plaur) of NGF-sensitive genes.

MATERIALS AND METHODS

Antibodies and Reagents

Polyclonal antibody to rat SH2B1 (αSH2B1), kind gift of Dr. Liangyou Rui (University of Michigan), was raised against an SH2B1β glutathione S-transferase fusion protein (91) and used at a dilution of 1:1000 for Western blotting. Monoclonal antibody to neuronal β-tubulin (TUJ1) was from Covance (Richmond, CA) (MMS-435P) and used at a dilution of 1:1000 for immunostaining of neurites. Antibody to α-tubulin (Cell Signaling Technology, Beverly, MA) was used at a dilution of 1:1000 for Western blotting. Polyclonal anti-MMP3 antibody (αMMP3) was from Chemicon (Temecula, CA; catalog no. AB19150) and used at 1:1000 for Western blotting. A polyclonal antibody against the ligand-binding NH_2-terminal domain of rat uPAR was used for Western blots at a dilution 1:500 as previously described (92). Antimouse uPAR monoclonal antibody used for immunostaining (at a dilution of 1:1000) was from R&D Systems (Minneapolis, MN; catalog no. AF534). Alexa Fluor 555-conjugated anti-mouse IgG used for immunocytochemistry and Alexa Fluor 680-conjugated anti-rabbit IgG used for Western blotting came from Invitrogen (Carlsbad, CA). NGF, rat-tail collagen I, and growth factor-reduced Matrigel were purchased from BD Bioscience, San Diego, CA. Purified human MMP3 was from Triple Points Biologics (Forest Grove, OR; catalog no. H-MMP-3, 0.1 μg/μl), fluorescein was from Bio-Rad (Hercules, CA) and SYBR Green I was from Sigma Chemical Co. (St. Louis, MO). Taq-Man RT-PCR kit was purchased from Applied Biosystems (Roche, Indianapolis, IN) (catalog no. N808-0234).

Stable Cell Lines and Cell Culture

The stock of PC12 cells was purchased from American Type Culture Collection (Rockville, MD). PC12 cells were plated on collagen-coated plates (0.1 mg/ml rat tail collagen in 0.02 N acetic acid) and grown at 37 C in 10% CO_2 in DMEM (Invitrogen) supplemented with 10% heat-inactivated horse serum (ICN, Aurora, OH), 5% fetal bovine serum (Invitrogen), 1 mM L-glutamine and 1 mM antibiotic-antimycotic (Invitrogen). PC12 cells stably overexpressing GFP (control PC12 cells), GFP-SH2B1β (PC12-SH2B1β cells), or GFP-SH2B1β(R555E) [PC12-SH2B1β(R555E) cells] were described previously (30). Experimental plates were incubated in serum-free medium overnight and then treated with vehicle or 100 ng/ml NGF for the indicated times.

RNA Preparation and Microarray Analysis

Total RNA was isolated from control and NGF-treated cells using Stat60 (Tel-Test, Inc., Friendswood, TX) and RNeasy spin columns (QIAGEN, Valencia, CA) according to the manufacturers' instructions. RNA from five experiments, each performed in duplicate, was prepared and the quality of RNA checked by OD and agarose gels. To assess further the quality of the RNA and confirm that the cells responded to NGF, QT-PCR was used to monitor the expression level of some known NGF-responsive genes. Based on these results, RNA from duplicates of two independent experiments was combined in equal proportions for microarray analysis. Increasingly, data sets in the Gene Expression Omnibus Database use pooled RNA samples for microarray analyses; the advantage of pooled RNA samples has been documented (93, 94). Preparation of cRNA from equal amounts of pooled RNA per cell line and its hybridization to rat genome RAE233A oligonucleotide arrays (Affymetrix, Santa Clara, CA) was performed by the Cell and Molecular Biology Core of the Michigan Diabetes Research and Training Center. The averaged NGF responsiveness (+NGF/−NGF) was used in Tables 1, 2, and 4 for genes represented by more than one probe set. Probe sets with raw data less than 100 in all six samples were not included in Tables 1–4 to minimize false-positive results. For calculating NGF responsiveness, values of raw data that are smaller than 50 are regarded as 50.

QT-PCR

Gene expression of Plaur, Mmp3, Mmp10, Stc1, Ca2, and Glrx1 was determined by QT-PCR using SYBR Green I and the iCycler system with iCycler iQ Real Time Detection System software (Bio-Rad). Primer sequences were designed using PrimerExpress software and are listed in Table 5. Amplicons generated from each primer pair were 50–52 bp. Loading of each sample was normalized with fluorescein. All

Table 5. Sequences of the QT-PCR Primers Used in This Study

Gene	Sequences (5′–3′)
Mmp3	
Forward	TGAAGATGACAGGGAAGCTGG
Reverse	GGCTTGTGCATCAGCTCCAT
Mmp10	
Forward	GAAATGGTCACTGGGACCCTC
Reverse	TGCGCAGCAACCAGGAATA
GAPDH	
Forward	ATGACTCTACCCACGGCAAGTT
Reverse	TCCCATTCTCAGCCTTGACTGT
Plaur	
Forward	ACAGGACCATGAGCTACCGC
Reverse	TCTCGGTGAGGCTGACGATC
Stc1	
Forward	AACATGGCCAGCCTCTTCC
Reverse	TGTCTGGGCACAGTGGTCTG
Glrx1	
Forward	AGCATGGCTCAGGAGTTTGTG
Reverse	CGACCACCTTTCCAGACTGAA
Ca2	
Forward	TGGACATTGACACCGGGACT
Reverse	GCAGAGGCTGTAGGGAAGGG

readings were normalized to the gene expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Levels of GAPDH expression were not different in control PC12, PC12-SH2B1β, and PC12-SH2B1β(R555E) cells (Figs. 3 and 4) or in shControl *vs.* PC12-shSH2B cells (Fig. 9B).

Cell Lysis and Immunoblotting

Cells were washed three times with chilled PBS [10 mM sodium phosphate, 137 mM NaCl, (pH 7.4)] containing 1 mM Na_3VO_4, solubilized in sample buffer [62.5 mM Tris-HCl (pH 6.8), 25% glycerol, 4% SDS, 0.01% bromophenol blue], and boiled for 10 min before centrifugation at 13,200 rpm for 1 min. Equal amounts of the solubilized proteins were separated on SDS-PAGE gels, transferred to polyvinylidene fluoride (Fig. 6A) or nitrocellulose (Fig. 9A) membranes, immunoblotted with the indicated antibody, and detected using Alexa Fluor 680-conjugated antirabbit IgG and an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

Analysis of uPAR Distribution

PC12 cells were plated onto Matrigel-coated coverslips, deprived of serum and incubated for the indicated time with 100 ng/ml NGF. Cells were fixed with 4% paraformaldehyde and incubated with uPAR monoclonal antibody followed by Alexa Fluor 555-conjugated secondary antibody. Analysis of uPAR distribution was performed with an Olympus FluoView 500 laser scanning confocal microscope using a ×60 oil-immersion objective and FluoView version 5.0 software. Alexa Fluor 555 fluorescence was excited with a green HeNe laser at 543 nm, and emission was measured through a 560-nm long pass filter (560 and above). GFP fluorescence was excited with an Ar laser at 488 nm, and emission was measured through a 505- to 525-nm filter.

Zymogram Gel Analysis

PC12 cells were incubated overnight in DMEM containing 0.1% chicken ovalbumin and then treated with 100 ng/ml NGF as indicated. Proteins in conditioned medium were concentrated 3- to 4-fold using Centricon 30 concentrator (Millipore, Bedford, MA) and separated on a 0.1% casein-containing, prestained, Tris-glycine gel (Invitrogen) under nonreducing conditions. The gel was washed three times with renaturation buffer (2.5% Triton X-100) to remove SDS and renature the MMPs and then developed overnight at 37 C in developing buffer [50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35] to visualize regions in the gel that contain active MMP.

Neurite Outgrowth through Matrigel

Neurite outgrowth through Matrigel was assessed using a modified method of Nordstrom *et al.* (36). Briefly, 50 μl of 1:3 diluted growth factor-reduced, phenol red-free Matrigel (BD Biosciences) was added to the *cis*-plane of a 24-well transwell membrane (6.5 mm^2, 5 μm pore size) and allowed to solidify at 37 C for 30 min. The *trans*-plane of the transwell membrane was coated with 1:10 diluted growth factor-reduced Matrigel overnight at room temperature. Cells were plated onto the *cis*-plane of the transwell membrane at a density of 50,000 cells per well in phenol red-free DMEM containing 0.1% chicken ovalbumin. NGF (100 ng/ml) was added to the outer chamber. After 4 d, cells were fixed and immunostained for neuronal β-tubulin (TUJ1) followed by Alexa Fluor 555-conjugated secondary antibody. Neurites on the *trans*-plane of the membrane were visualized by fluorescence microscopy (Nikon Eclipse TE2000) and counted.

Silencing of SH2B1 Gene

SH2B1 small interfering RNA (siRNA) vector was constructed by inserting an oligonucleotide containing the SH2B1 sequence (5′-CATCTGTGGTTCCAGTCCA-3′) corresponding to nucleotides 1771–1789 of rat *SH2B1* (GenBank accession no. AF047577) into the pSuper retro vector containing the puromycin resistance gene (pSuper retro puro) (OligoEngine, Seattle, WA). A pSuper vector containing a nontargeting siRNA with a low sequence similarity to known genes was used as a control. The SH2B1 and control siRNA vectors were transfected into subconfluent PC12 cells using a Bio-Rad Gene Pulser Xcell electroporator (400V, 500 μF, 0.4-cm cuvette). After 14 h, cells were washed with PBS, and fresh growth medium was added. Twenty-four hours later, the medium was changed to a selection medium containing 5 μg/ml puromycin and selected for pSuper-positive PC12 cells for 30 d. The efficacy of the SH2B1 RNAi was assessed using QT-PCR on RNA from the puromycin-resistant PC12 lines (data not shown).

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The unprocessed microarray data are accessible through GEO series accession no. GSE4557 in the NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo).

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