The Non-transmembrane Form of Delta1, but Not of Jagged1, Induces Normal Migratory Behavior Accompanied by Fibroblast Growth Factor Receptor 1-dependent Transformation*

Received for publication, December 29, 2003, and in revised form, January 29, 2004
Published, JBC Papers in Press, February 9, 2004
DOI 10.1074/jbc.C300564200

Radiana Trifonova, Deena Small, Doreen Kacer, Dmitry Kovalenko, Vihren Kolev, Anna Mandinova, Rassafa Soldi, Lucy Liaw, Igor Prudovsky, and Thomas Maciag†

From the Center for Molecular Medicine, Maine Medical Center Research Institute, Scarborough, Maine 04074

The interactions between Notch (N) receptors and their transmembrane ligands, Jagged1 (J1) and Delta1 (Dl1), mediate signaling events between neighboring cells that are crucial during embryonal development and in adults. Since the non-transmembrane extracellular form of J1 acts as an antagonist of N activation in NIH 3T3 mouse fibroblast cells and induces fibroblast growth factor factor 1 (FGF1)-dependent transformation (Small, D., Kovalenko, D., Soldi, R., Mandinova, A., Kolev, V., Trifonova, R., Bagala, C., Kacer, D., Battelli, C., Liaw, L., Prudovsky, I., and Maciag, T. (2003) J. Biol. Chem. 278, 16405–16413), we examined the potential redundant functions of the two subfamilies of Notch ligands and report that while the soluble (s) forms of both Dl1 and J1 act as N signaling antagonists in NIH 3T3 cells, they do display disparate functions. While sJ1 induced an attenuation of cell motility which is accompanied by a decrease in actin stress fibers and an increase in adherence junctions, sDl1 does not. However, sJ1, like sDl1, induces a NIH 3T3 cell transformed phenotype mediated by FGF signaling. Because the inhibition of N signaling by sJ1 and sDl1 is rescued by dominant-negative Src expression, we suggest that there may be cooperation between the Notch and Src signaling pathways.

* This work was supported in part by National Institutes of Health Grants HL70865 (to L. L.) and HL35627, HL32348, and RR15555 (to T. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† This work was performed by R.T. in partial fulfillment of the requirements for the Ph.D. from the University of Sofia, Bulgaria.

‡ Current address: Dept. of Animal Nutritional and Medical Laboratory Sciences, College of Life Sciences and Agriculture, University of New Hampshire, Durham, NH 03824.

§ Current address: Cutaneous Biology Research Center, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA 02129.

To whom correspondence should be addressed: Center for Molecular Medicine, Maine Medical Center Research Inst., 81 Research Dr., Scarborough, ME 04074. Tel.: 207-885-8200; Fax: 207-885-8179; E-mail: maciat@mmc.org.

The Notch gene family encodes evolutionarily conserved cell surface receptors that initiate signaling between neighboring cells in multicellular organisms. Notch signaling plays a role in cell fate determination through the regulation of cell proliferation, cell differentiation, and apoptosis. Aberrant Notch signaling is associated with developmental defects and pathologic conditions in adults.

The Notch receptors and their ligands are single-pass transmembrane proteins with conserved protein structures. The ligands for the Notch receptors have traditionally been divided into two subclasses, Delta-like and Serrate-like, defined by the absence and presence, respectively, of an additional cysteine-rich domain in the extracellular portion of the polypeptide. The mechanism of Notch receptor signaling has been extensively studied and involves the activation of CSL (for CBP in mammals, Suppressor of Hairless (Sco(H)) in Droso phila and Xenopus and Lag-1 in Caenorhabditis elegans) dependent transcription mediated by the nuclear translocation of the intracellular domain of Notch.

The existence of soluble forms of Notch ligands including the extracellular portion of Delta in Droso phila and other organisms have been reported but their physiological roles have not been determined. Transcripts encoding the extracellular domain of the Jagged1 ligand have also been detected in human endothelial cells. There is evidence that the Delta ligand can be proteolytically cleaved by Kuzbanian, a member of the ADAM family metalloproteases, to generate a soluble extracellular form, and recent evidence also suggests that Notch ligands may be processed by the γ-secretase presenilin in a manner similar to the Notch receptor.

While studies have suggested that the soluble forms of the Notch ligands are able to activate Notch receptors, there are numerous reports that the soluble forms of the Notch ligands act as antagonists of Notch signaling by impeding the interaction between Notch receptors and their full-length ligands. Secreted forms of Delta perturb association between full-length Delta and Notch and inhibit the Notch-dependent repression of myoblast (23) and hematopoietic progenitor cell (14) differentiation in vitro. Likewise, the expression of a non-transmembrane form of the Notch ligand, Jagged1 (sJ1) also antagonizes Notch signaling in NIH 3T3 cells and induces significant changes in their cellular phenotype including FGFRI-dependent transformation (10, 24). Thus, to determine whether the secreted form of the Delta1 ligand would generate effects exhibited by the non-transmembrane form of Jagged1, we investigated the phenotypic characteristics of NIH 3T3 cells stably transfected with the soluble form of the Delta1 (sDl1) ligand.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—Soluble Dl1 expressing stable cell lines were created by transfecting mouse fibroblast NIH 3T3 cells with pCDNA3.1(+), in which the DNA sequence encoding the human D1 tag.
extracellular domain was inserted in-frame with a Myc His tag (14) and the positive clones selected by Geneticin resistance as described previously (10). The stable sJ1 and the dn-src NIH 3T3 cell transfectants as well as the cell culture conditions to grow and maintain these transfectants were described previously (10). Experiments were performed with multiple sD1 NIH 3T3 cell clones and we observed no significant differences between clones expressing either high or low levels of the sD1 protein.

Cell Migration Assay—Approximately 10^5 sJ1 or sD1 NIH 3T3 cell transfectants were plated in DMEM supplemented with 10% (w/v) bovine calf serum (BCS; Hyclone), and after the cells reached ~60–70% confluence, the cell monolayer was scratched using a plastic cell scraper. Photomicrographs were taken immediately after the injury and 24 and 48 h later.

Immunofluorescence Microscopy—Cells were plated on glass coverslips and fixed with 4% (v/v) paraformaldehyde. Actin stress fibers were visualized by fluorescein isothiocyanate-phalloidin (Sigma), a monoclonal anti-vinculin antibody (Sigma) was used to visualize focal adhesion sites, and a monoclonal anti-beta-catenin antibody (BD Transduction Laboratories) and a monoclonal anti-pan-cadherin antibody (Sigma) were both used to visualize cell-cell adhesions as described previously (10).

Immunoblot Analysis—Cell lysates from insert-less vector sD11 and sJ1 NIH 3T3 cell transfectants were prepared and immunoblot analysis was performed as described previously (10) using either a rabbit anti-src (pY418) phosphospecific antibody (BIOSOURCE International), a rabbit anti-total Src antibody (Santa Cruz Biotechnology), a rabbit polyclonal anti-cortactin antibody, or an anti-phosphothreonine antibody (Upstate Biotechnology). Immunoprecipitation of cortactin was performed as described previously (25).

RESULTS AND DISCUSSION

We have previously demonstrated that the expression of a soluble form of Jagged1 (sJ1) induced significant changes in the phenotype of NIH 3T3 cells including the onset of a FGFR1-dependent phenotype with impaired cell motility, associated with reduced actin stress fibers and focal adhesion sites formation (24). To determine whether these events were sJ1-specific, we examined the phenotype of NIH 3T3 cells stably transfected with soluble extracellular domain of Delta1 (sD11). Interestingly, although the stable sD11 and sJ1 NIH 3T3 cell transfectants shared some characteristics, there were significant differences between them; the most striking being their migratory behavior. Indeed, as shown in Fig. 1A, the sD11 NIH 3T3 cell transfectants displayed normal motility, while the sJ1 cells exhibited a decrease in their migratory potential.

Since cell motility is dependent on F-actin stress fiber formation as well as cell-matrix and cell-cell adhesions, we examined the sD11 and sJ1 NIH 3T3 cell transfectants for these cellular features. While we observed normal levels of F-actin stress fibers and focal adhesion sites in the sD11 NIH cell...
transfectants, the s1 NIH 3T3 cell transfectants exhibited significantly reduced actin stress fibers and focal adhesion sites (Fig. 1B). Furthermore, as shown in Fig. 1B, the sD1 NIH 3T3 cell transfectants did not display an increase in cell-cell adhesion junctions, as revealed by β-catenin and pan-cadherin immunofluorescent staining, which was prominently exhibited in the sJ1 NIH 3T3 cell transfectants (Fig. 1B). These data suggest that the sD1 NIH 3T3 cell transfectants exhibited cellular architectural properties consistent with a normal motility phenotype, whereas the sJ1 NIH 3T3 cell transfectants did not.

To further study the differences in the organization of the F-actin cytoskeleton in the sD1 and sJ1 NIH 3T3 cell transfectants, we compared the levels of the activated form of Src and its substrate cortactin, which is known to play a role in the organization of the cortical actin network and F-actin stress fibers (26). As shown in Fig. 2, we observed a decrease in the level of tyrosine-phosphorylated cortactin in sD1 NIH 3T3 transfectants that contrasted with the increased level observed in the sJ1 NIH 3T3 cell transfectants. Since the tyrosine phosphorylation of cortactin exhibits low affinity for F-actin, which results in an attenuation of F-actin filament bundling, this observation is consistent with the appearance of F-actin stress fibers in the sD1 NIH 3T3 cell transfectants and a reduction in the appearance of F-actin stress fibers in the sJ1 NIH 3T3 cell transfectants (Fig. 1B). However, because the sD1 and sJ1 NIH 3T3 cell transfectants both exhibited an increase in the level of active Src (Fig. 2), it may be possible that the differential phosphorylation of cortactin in these transfectants may be related to the ability of the sD1 NIH 3T3 cell transfectants to uncouple the association between Src and cortactin. While it is known that sJ1 NIH 3T3 cell transfectants enable Src to associate with cortactin (10), cortactin immunoprecipitation followed by either Src or phosphotyrosine immunoblot analysis failed to resolve Src from cell lysates derived from the sD1 NIH 3T3 cell transfectants (data not shown).

Despite the differences in the migratory behavior, F-actin cytoskeleton organization and the abundance of focal adhesion sites between the sD1 and sJ1 NIH 3T3 cell transfectants, these cells did exhibit a variety of common characteristics including accelerated proliferation at high cell density and anchorage-independent growth in soft agar. Growth kinetics of both sD1 and sJ1 NIH 3T3 cell transfectants revealed impaired contact inhibition of growth as compared with the insert-less vector control transfectants (Fig. 3A). In addition, the sD1 and sJ1 NIH 3T3 cell transfectants were also able to grow in an anchorage-independent manner and form colonies in soft agar (Fig. 3B). Furthermore, the size but not the number of colonies was significantly exaggerated upon the addition of exogenous FGF1, suggesting a synergy between FGF1 and the soluble forms of the Notch ligands. The accelerated proliferation at high cell density of both sD1 and sJ1 NIH 3T3 cell transfectants may be attributed to the function of Src, since expression of a dnSrc construct in both the sD1 and sJ1 NIH 3T3 cell transfectants restored contact inhibition of cell growth (Fig. 3A).

In contrast, however, the anchorage-independent growth of both the sD1 and sJ1 NIH 3T3 cell transfectants appeared to be independent of the function of Src, since the sD1/dnSrc and sJ1/dnSrc NIH 3T3 cell co-transfectants still formed colonies in soft agar (Fig. 3B). These data suggest that the non-transmembrane forms of the Notch ligands, Jagged1 and Delta1, utilize similar pathways to regulate NIH 3T3 anchorage-independent and independent cell growth.

Because the NIH 3T3 cell responds to sJ1 as a repressor of CSL-dependent Notch signaling (10, 24), we sought to determine whether sD1 was also able to modify CSL-dependent signaling in the NIH 3T3 cell and whether the expression of...
dnSrc rescued this response. As shown in Fig. 4, we observed that like sJ1, the expression of sD11 also inhibited the transactivation of the CBF1-responsive promoter in both NIH 3T3 cells and NIH 3T3 cells stably transfected with the constitutively active intracellular domain of Notch (N1IC). Interestingly, the expression of dnSrc in either the N1IC background or in wild type NIH 3T3 cells rescued the repression of CBF1-dependent transcription by both sJ1 and sD11 (Fig. 4). These data suggest that Src may be a component of the signaling pathway utilized by both sJ1 and sD11 to repress the N1IC-dependent transcription, and this is consistent with the levels of functional Src observed in both the sJ1 and sD11 NIH 3T3 cell transfectants (Fig. 2). Indeed, this is to our knowledge the first evidence suggesting an association and/or cross-talk between CSL-mediated Notch signaling and Src. Although the mechanism responsible for this event remains to be determined, it is possible that Src may interfere with the formation of the CSL-Notch1 intracellular domain complex. Because expression of a constitutively active Src mutant in NIH 3T3 cells also results in the repression of CBF1-dependent Notch signaling, yet the expression of dnSrc in the NIH 3T3 cell activates CSL-dependent Notch signaling (Fig. 4), it is likely that the activation of Src by either sJ1 or sD11 may establish a negative loop between active Src and a component of the Notch signaling pathway.

It is noteworthy that while CSL transactivation is rescued in the sD11/dnSrc and sJ1/dnSrc NIH 3T3 cell co-transfectants, these cells remain able to form colonies in soft agar. This observation suggests that the anchorage-independent growth of the sD11 and sJ1 NIH 3T3 cell transfectants is not only Src-independent but also CSL-independent. While the constitutive, non-stress-dependent FGF1 release pathway is functional in the sD11 NIH 3T3 cell transfectants, analysis by reverse transcription-PCR suggests that sD11, unlike sJ1 (24), is unable to induce the expression of the FGF1 transcript (data not shown). However, while the FGF1, FGF3, FGF4, and FGF5 transcripts are induced by the expression of sJ1 in NIH 3T3 cells (24), sD11 NIH 3T3 cell transfectants did not exhibit the presence of the FGF1 and FGF5 transcripts; rather, the sD11 NIH 3T3 cell transfectants express the FGF3, FGF4, and FGF9 transcripts (data not shown). Since FGF1 signaling is able to activate Src in NIH 3T3 cells (27), it is possible that FGF4- and/or FGF9-mediated FGF1 signaling may not only be involved in the activation of Src in the sD11 NIH 3T3 cell transfectants but may also enable the sD11 and dnSrc NIH 3T3 cell co-transfectants to survive and grow, albeit at a reduced level, both as monolayer and in soft agar in vitro (Fig. 3, A and B). This premise is supported by the observation that the anchorage-independent growth of the sD11 NIH 3T3 cell transfectants (Fig. 3B) was completely inhibited by the addition of the FGF1-specific inhibitor, PD168666 (data not shown).

While these data suggest that sD11 and sJ1 modify similar growth-related phenotypes in the NIH 3T3 cells, the expression of these soluble Notch ligands do exhibit disparate phenotypic characteristics most notably in the regulation of cell migration in vitro. These differences could be due to differential interactions with the Notch receptors, since Jagged1, but not Delta1, contains both an additional cysteine-rich motif and a higher number of epidermal growth factor-like repeats within the extracellular domain (6). It is also possible that these differences may be due to other factors such as the function of the glycosyltransferase Fringe (28), which may influence ligand binding. In addition, since the NIH 3T3 cells express only the Notch1 and Notch2 transcripts (data not shown), the soluble forms of the Delta1 and Jagged1 may be able to either antagonize different Notch receptors with different affinity or perhaps interfere with the activation of the Notch receptors with a different efficiency even though both are able to repress CSL-dependent signaling to a similar extent. However, because we did not observe differences among multiple stable sD11 NIH 3T3 cell transfectants expressing different levels of sD11, it is unlikely that the differences between the expression levels of sD11 and sJ1 account for these disparate behaviors.

There is also evidence suggesting that Notch receptor-ligand interactions may trigger bi-directional signaling (29, 30). Indeed, the intracellular portion of the Jagged1 transmembrane protein does contain a PDZ-domain, which may be able to mediate interactions with other signaling systems regulated by SH2 domain-containing proteins (30) such as Src. Since Jagged1, but not Delta1, is expressed endogenously in NIH 3T3 cells, it is intriguing to suggest that the differences in NIH 3T3 cell migratory behavior exhibited by the expression of sJ1 and sD11 may be the result of the ability of sD11 to modify a Jagged1-dependent, PDZ-domain-regulated signal.

Acknowledgments—We thank Drs. R. L. Panek (Parke-Davis) for the FGFR1-specific inhibitor, PD168666, R. Friesel (Maine Medical Center Research Institute) for the mutant, M. A. Moore (Memorial Sloan- Kettering Cancer Center) for the delta1 cDNA, and X. Zhan (American Red Cross) for the cortactin antibody. We also thank Norma Albrecht and Barbara Peaselee for expert administrative assistance.

REFERENCES

1. Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999) Science 284, 770–776
2. Jonas, S., and Capobianco, A. J. (2000) Mol. Cell. Biol. 20, 3928–3941
3. Xue, Y., Gao, X., Lindsell, C. E., Norton, C. R., Chang, B., Hicks, C., Gendron-Maguire, M., Rand, B. R., Weinmaster, G., and Gridley, T. (1999) Hum. Mol. Genet. 8, 723–730
4. Jouet, A., and Tournaire-Lassere, E. (1998) Semin. Cell Dev. Biol. 9, 619 – 625
5. Lardelli, M., Dahlstrand, J., and Lendahl, U. (1994) Mech. Dev. 46, 123–136
6. Weinmaster, G. (1998) Curr. Opin. Genet. Dev. 8, 436–442
7. Ziman, A. R., Pepper, M. S., Nguyen, F., Montesano, R., and Raso, M. (1997) J. Biol. Chem. 272, 32499–32502
8. Jarzab, S., Brou, C., Logeat, F., Schroeter, E. H., Kopan, R., and Israel, A. (1995) Nature 377, 355–358
9. Zhou, S., Fujimura, M., Hsieh, J. J., Chen, L., Miyamoto, A., Weinmaster, G., and Hayward, S. D. (2000) Mol. Cell. Biol. 20, 2400–2410
10. Small, D., Kovalenko, D., Kacer, D., Law, L., Landrisina, M., Di Serio, C., Prudovski, I., and Macagno, T. (2001) J. Biol. Chem. 276, 32022–32030
11. Nofziger, D., Miyamoto, A., Lyons, K. M., and Weinmaster, G. (1999) Develop. (Camb.) 126, 1689–1700
12. Frankie, L. L., Berechid, B. E., Cutting, B. F., Presente, A., Chambers, C. B., Fultz, D. R., Ferreira, A., and Nye, J. S. (1999) J. Biol. Chem. 274, 4427–4437
13. Han, W., Ye, Q., and Moore, M. A. (2000) Blood 95, 1616–1625
14. Kluger, R. M., Parody, T. R., and Muskavitch, M. A. (1998) Mol. Cell. Biol. 19, 1709–1723
15. Husriede, N. A., Yu, G., and Fleming, R. J. (1997) Develop. (Camb.) 124, 3427–3437
16. Cooper, J. A., and Parsons, J. T. (2000) J. Biol. Chem. 275, 7751–7754
The Non-transmembrane Form of Delta1, but Not of Jagged1, Induces Normal Migratory Behavior Accompanied by Fibroblast Growth Factor Receptor 1-dependent Transformation

Radiana Trifonova, Deena Small, Doreen Kacer, Dmitry Kovalenko, Vihren Kolev, Anna Mandinova, Raffaella Soldi, Lucy Liaw, Igor Prudovsky and Thomas Maciag

J. Biol. Chem. 2004, 279:13285-13288.
doi: 10.1074/jbc.C300564200 originally published online February 9, 2004

Access the most updated version of this article at doi: 10.1074/jbc.C300564200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 20 of which can be accessed free at http://www.jbc.org/content/279/14/13285.full.html#ref-list-1