Activity of Wnt-1 as a transmembrane protein

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The product of the Wnt-1 proto-oncogene is a cysteine-rich glycoprotein that plays a crucial role in the development of the vertebrate central nervous system. Wnt-1 protein is secreted but remains associated with the cell surface and extracellular matrix. The function of Wnt-1 in several different biological settings can be carried out by cells that receive the Wnt signal from adjacent cells. Ectopic expression of Wnt-1 in certain mammary gland cell lines, such as C57MG, causes morphological transformation; C57MG cells can also be transformed by a paracrine mechanism when mixed with other cell types secreting Wnt-1 protein. To ask whether Wnt-1 protein can function while bound to the cell of origin, a variety of cell types were programmed to produce chimeric proteins containing the complete sequence of mature Wnt-1 protein fused to part or all of the transmembrane protein CD4 or CD8. The chimeras were found at the cell surface of transfected cells and did not appear to be proteolytically processed. In autocrine and paracrine transformation assays with C57MG cells and in an axis induction assay in Xenopus laevis embryos, the Wnt-1/CD4 or CD8 fusions retained significant activity, as did a secreted chimera containing the CD8 extracellular domain but lacking the transmembrane domain. However, a chimera lacking a spacer between the Wnt-1 and the transmembrane domains was weakly active and only in autocrine transformation. These results show that tethering Wnt-1 to the cell surface still allows Wnt-1-mediated cell-to-cell signaling.

[Key Words: Wnt-1; transmembrane protein; transformation; mammary oncogene; CD4; CD8]

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is limited to a single band of cells in each segment; en expression is regulated in an immediately adjacent band of cells. Immunoelectron microscopy has demonstrated the presence of wg protein inside cells that express en, up to several cells away from the wg-expressing cells (van den Heuvel et al. 1989; González et al. 1991). Genetic analysis of wg transcription strongly suggests that wg acts via a paracrine, rather than autocrine, mechanism (Ingham and Hidalgo 1993).

In X. laevis embryos, injection of several different Wnt RNAs (including wg, murine Wnt-1, and Xenopus Wnt-1, 3A, and 8) leads to the induction of a new Spemann organizer and formation of a second body axis (Moon 1993). Deletion of the hydrophobic leader sequence or mutation of a critical cysteine residue, C369, inactivates the ability of Wnt-1 to induce a secondary axis (McMahon and Moon 1989). The Wnt-injected cells themselves do not necessarily populate the new organizer region or body axis, indicating that they can act as donors of an inducing signal to surrounding cells. Thus, it has been proposed (Smith and Harland 1991; Sokol et al. 1991) that an endogenous Wnt protein is the signal that emamates from the Nieuwkoop center (Kimelman et al. 1992). It is also possible, however, that the Wnt-expressing cells are induced to secrete secondary factors that are directly responsible for organizing the body plan.

Wnt-1 protein is capable of transforming the morphology of the murine mammary gland cell line C57MG by a paracrine mechanism (Jue et al. 1992, Mason et al. 1992). Wnt-1-expressing cells, such as rat or mouse fibroblasts that show no detectable response themselves, can transform neighboring C57MG cells in coculture experiments. This activity depends on entry of the Wnt-1 protein into the secretory pathway, because deletion of the signal sequence abolishes transforming activity (Mason et al. 1992). The distance over which Wnt-1 may act has been estimated by performing paracrine assays using as donors small colonies of Wnt-1-expressing rat (Jue et al. 1992) or QT6 cells (N. Parkin, J. Kitajewski, and H.E. Varmus, unpubl.). In such experiments, surrounding C57MG cells show morphological transformation in a ring ~5-10 cells wide.

In cultured cells expressing Wnt-1 ectopically, the majority of Wnt-1 protein is present in the endoplasmic reticulum (ER), where it associates with the immunoglobulin heavy chain-binding protein BiP (Kitajewski et al. 1992); only a relatively small proportion is actually secreted. Once outside the cell, Wnt-1 protein associates tightly with the cell surface and the extracellular matrix (ECM), where it can be released by incubation with suramin or heparin (Bradley and Brown 1990; Papkoff and Schryver 1990). Such treatment, however, abrogates its activity. Proteins encoded by other members of the Wnt gene family behave in a similar fashion (Blasband et al. 1992; Chakrabarti et al. 1992; J. Kitajewski, N. Parkin, and H.E. Varmus, unpubl.). In this way, the range of action of Wnt protein can be restricted, implying that the Wnt signal may be confined to cells only a short distance away from the donor cell in vivo. Severe defects in segmental patterning are observed when wg is expressed ubquitously in Drosophila embryos (Noordermeer et al. 1992), implying that restriction of the range of action of wg protein is important for segment polarity.

Thus, the biochemical and genetic properties of Wnt-1 suggest that it is a locally acting growth factor and that mechanisms for restriction of its range of action are inherent in these properties. Another potential mechanism for limiting the range of action of a growth or differentiation factor is its expression as a transmembrane protein. Several other growth factors have been described that can function as transmembrane proteins (Brachmann et al. 1989; Mroczkowski et al. 1989; Wong et al. 1989; Perez et al. 1990; Dobashi and Stern 1991; Krämer et al. 1991; Rebay et al. 1991). To determine whether or not Wnt-1 can function as a transmembrane rather than a secreted protein, we generated chimeric proteins between Wnt-1 and two transmembrane molecules, CD4 and CD8. The transmembrane fusions are active in transformation assays in C57MG cells and induce a new body axis in X. laevis. Although the activity of the chimeras is less than that of wild-type Wnt-1, it is similar to that of a secreted chimera containing only the extracellular domain of CD8. We conclude that conversion of Wnt-1 into a transmembrane protein does not in itself impair biological activity.

Results

Wnt-1/CD4 and CD8 chimeras

Two well-characterized transmembrane proteins, CD4 and CD8, were chosen to construct Wnt-1-containing chimeric proteins anchored to cell membranes. These proteins are involved in the T-cell activation response and are thought to interact with major histocompatibility complex [MHC] class I (CD8) or class II (CD4) proteins on the surface of antigen-presenting cells (Janeway 1992). Both CD4 and CD8 are thought to have a rigid, rod-like structure that extends the MHC-binding sites away from the cell membrane. Therefore, the Wnt-1 portion of WntCD4 and WntCD8 chimeras should be able to bind to the Wnt-1 receptor on adjacent responsive cells.

Fusions were made to the carboxyl terminus of the Wnt-1 protein, because we have previously added amino acids there without loss of Wnt-1 activity (J. Kitajewski, J. Mason, and H.E. Varmus, unpubl.). The complete Wnt-1-coding sequence was used to preserve the penultimate amino acid, a cysteine at position 369 (C369), known to be critical for Wnt-1 function (McMahon and Moon 1989; Mason et al. 1992). In constructs containing the entire extracellular domain of CD4 or CD8, the junctions are at the predicted signal peptide cleavage sites of the transmembrane protein (e.g., WntCD4, WntCD8, and WntCD8s, see Fig. 1). A shorter construct, containing only a 5-amino-acid spacer between the carboxyl terminus of Wnt-1 and the transmembrane domain of CD4, was also made [WntΔCD4]. To examine possible effects of the extracellular domain of CD8 on the activity of Wnt-1, a secreted fusion lacking the transmembrane and cytoplasmic sequences [sWntCD8] was created by intro-
Activity of Wnt-I as a transmembrane protein

Figure 1. A schematic representation of Wnt-I and derived transmembrane chimeras. Constructs were assembled in pBlue-script plasmids and transferred to pLHTCX for generation of recombinant retrovirus stocks or to pSP64T for in vitro transcription. The extracellular domains of CD4 and CD8 are 374 and 161 amino acids in length, respectively. WntACD4 retains only 43 amino acids of the CD4 extracellular domain. For details of construction, see Materials and methods. (tm) Transmembrane domain. The signal peptide is represented by a black box.

Reducing a stop codon just 5' of the start of the region encoding the CD8 transmembrane domain. In WntCD8ζ the cytoplasmic portion of CD8 has been exchanged for that of the T-cell receptor signaling molecule, ζ (Irving and Weiss 1991; Weiss 1993). This chimera was made primarily for other purposes (see Discussion) but serves here as a control for any potential effects of the cytoplasmic portion of the WntCD8 fusion. Also, the inactivating C369W mutation (McMahon and Moon 1989; Mason et al. 1992) was introduced in this background as a negative control.

Expression of Wnt-I/transmembrane chimeras

The various cDNA constructs were cloned into a retroviral expression vector and introduced into RatB1a cells. To ensure that intact fusion proteins were produced and not subsequently cleaved, clones of infected cells were pulse labeled for 1 hr with [35S]cysteine and incubated for 4 hr in medium containing excess unlabeled cysteine and 1 mM suramin (suramin is required to immunoprecipitate secreted Wnt-1 protein from the medium of expressing cells; Papkoff and Schryver 1990). As shown in Figure 2, only Wnt-1 and sWntCD8 could be detected in the medium after the chase. The apparent secretion efficiency of Wnt-1 is high in this cell line; however, this type of analysis tends to exaggerate the relative amount of labeled protein outside the cell, because the half-life of extracellular Wnt-1 protein is longer (Bradley and Brown 1990; Papkoff and Schryver 1990). The amount of sWntCD8 protein detected in the medium after the chase was ~15-fold lower (accounting for differences in cysteine content) than the amount of Wnt-1 protein. This may be the result of reduced secretion efficiency, a shorter extracellular half-life of this protein, or both. The sWntCD8 proteins found in the medium migrate significantly more slowly than the intracellular forms, and the shift in mobility is greater than that seen for Wnt-1. This effect is likely the result of O-linked glycosylation on the CD8 portion of the chimera (Snow et al. 1984). Importantly, after exposure of the autoradiograph for 4 days (Fig. 2), no Wnt-containing cleavage products could be detected in medium from any of the cell lines expressing the Wnt-1/transmembrane chimeras. After a 26-day exposure (40 times the minimum exposure needed to detect the sWntCD8 band), there were still no potential cleavage products visible, at this level of sensitivity, we would thus expect to be able to detect levels of Wnt-1-containing proteins as low as 40-fold below that of sWntCD8. After this long exposure, a faint band was visible in the last lane of the gel, possibly representing intact WntCD4 protein released into the medium in association with cellular debris. Scintillation counting of this region of the dried gel suggested that it represented at least 80-fold less protein than sWntCD8 (data not shown). This low level of protein in the medium can be considered insignificant in the context of the activity assays used (see below).

Wnt-1/transmembrane chimeras are expressed at the cell surface

To ensure that the transmembrane Wnt fusions were transported to the cell surface, RatB1a cell lines express-
Expression of Wnt-1/transmembrane chimeras in RatBla cells. Clonal cell lines of RatBla were generated and labeled with $[^{35}S]$cysteine for 1 hr and chased for 4 hr in complete medium containing 1 mM suramin. The chase medium or cell lysates were immunoprecipitated with anti-Wnt-1 monoclonal antibody and analyzed by electrophoresis and fluorography. Exposure times for the lysates (left) and chase medium (right) were 16 hr and 4 days, respectively.

Figure 2. Expression of Wnt-1/transmembrane chimeras in RatBla cells. Clonal cell lines of RatBla were generated and labeled with $[^{35}S]$cysteine for 1 hr and chased for 4 hr in complete medium containing 1 mM suramin. The chase medium or cell lysates were immunoprecipitated with anti-Wnt-1 monoclonal antibody and analyzed by electrophoresis and fluorography. Exposure times for the lysates (left) and chase medium (right) were 16 hr and 4 days, respectively.

The recombinant proteins were subjected to flow cytometry using fluorescently labeled monoclonal antibodies against CD4 or CD8. As shown in Figure 3, RatBla cells expressing the WntCD4 chimera were significantly more fluorescent than parental RatBla cells after addition of anti-CD4 antibody. The increase in fluorescence was not as great as that seen with RatBla cells expressing a control transmembrane protein, CD8\textsubscript{8} (Irving and Weiss 1991), stained with anti-CD8 antibody. If the relative levels of expression of the two proteins are equal, this observation suggests that transport of WntCD4 to the cell surface is relatively inefficient, as is secretion of wild-type Wnt-1 (Papkoff and Schryver 1990). Staining of cells expressing WntCD8 or WntCD8\textsubscript{8} with anti-CD8 did not result in a significant increase in fluorescence, partly because the antibody reacts poorly with the chimeras (the epitope recognized by the Leu2a monoclonal antibody may be partially masked), and partly because these chimeras are less efficiently transported to the cell surface (see below).

We then used indirect immunofluorescence methods to examine the appearance of chimeric proteins on the cell surface in more detail and to compare cells expressing the chimeric proteins with those expressing wild-type Wnt-1. Uninfected RatBla cells or clones expressing the various constructs were plated on fibronectin-coated coverslips and stained, unfixed and unpermeablized, with anti-Wnt monoclonal antibody (Fig. 4). In wild-type Wnt-1-expressing cells, Wnt-1 protein was detected on the extracellular matrix surrounding the cells, with some staining also seen over their surface (Fig. 4A,B). In contrast, RatBla cells expressing WntACD4 (Fig. 4C,D), WntCD4 (E,F), WntCD8 (G,H), or WntCD8\textsubscript{8} (not shown) showed a different type of staining. The observable staining was confined to the cell surface, implying little or no association with the ECM. Although the sensitivity of this procedure might not be sufficient to detect small amounts of secreted or cleaved WntCD4 or WntCD8 protein, when taken together with the immunoprecipitation analysis (Fig. 2) the data strongly indicate that the fusion proteins are confined to the cell surface. Cells
Activity of Wnt-1 as a transmembrane protein

Figure 4. Immunofluorescence analysis of cells expressing chimeric Wnt-1/transmembrane proteins. Uninfected RatBla cells (I,J) or clones expressing Wnt-1 (A,B), WntACD4 (C,D), WntCD4 (E,F), WntCD8 (G,H) or CD8~ (K,L) were plated on fibronectin-coated coverslips and incubated with anti-Wnt-1 (A–F) or anti-CD8 (OKT8) (K,L) monoclonal antibodies, followed by staining with Texas Red-conjugated anti-mouse IgG. Cells were photographed using Kodak Tri-X film at a magnification of ~500×. Immunofluorescence (IF) is shown on the left (A,C,E,G,I,K), and the corresponding phase contrast field (PHASE) is on the right (B,D,F,H,J,L).

expressing WntCD4 reproducibly gave the strongest signal, suggesting that the WntCD4 protein was the one most efficiently transported to the surface in this cell type. The low intensity of staining seen for WntACD4 is the result, in part, of a lower level of expression in this clone (see Fig. 2). Very low background staining was observed with parental RatBla cells (I,J). An intense pattern typical for abundant cell surface proteins was found after the staining of RatBla cells producing CD8~ with anti-CD8 antibody (K, L).

Activity of Wnt-1/transmembrane chimeras in C57MG cells

To test the chimeric transmembrane proteins for transforming activity, the recombinant constructs were introduced into the Wnt-1-responsive mammary gland cell line C57MG (Table 1; Fig. 5). As production of Wnt-1 protein in this cell line after retrovirus infection is somewhat unstable [Mason et al. 1992], all lines used to assay morphological changes were monitored for Wnt-1 protein by immunoblotting (Fig. 5; data not shown). Uninfected C57MG cells exhibit a flat, cuboidal type of morphology (Fig. 5A). As described previously [Brown et al. 1986], when C57MG cells produce Wnt-1, they are trans-

| Construct              | Activitya |
|------------------------|-----------|
|                        | autocrine | paracrine |
| Wnt-1                  | + + +     | + + +     |
| C369W                  | – – –     | – – –     |
| WntACD4                | +/– –     | – – –     |
| WntCD4                 | + + +     | + + +     |
| WntCD8                 | + + +     | + + +     |
| WntCD8~                | + + +     | + + +     |
| WntCD8~–C369W          | – – –     | – – –     |
| sWntCD8                | + + +     | + + +     |
| CD8~                   | – – –     | – – –     |

* For determination of autocrine activity, pools of C57MG cells producing the indicated protein were plated in 60-mm petri dishes, the medium was changed to HB-CHO at subconfluence, and the morphology was assessed 2 days later (see Materials and methods). For paracrine assays, clones of RatBla cells producing the chimeras were cocultured with C57MG cells at an equal ratio; again the medium was changed to HB-CHO at subconfluence. The degree of morphological transformation is indicated as follows: (−) Not detectable [e.g., Fig. 5A]; (+/–) weak but detectable [Fig. 5D]; (+) intermediate [e.g., Fig. 5C]; (++) morphology of wild-type Wnt-1-transformed C57MG cells [Fig. 5B].
Figure 5. Autocrine transformation assays in C57MG cells. Uninfected C57MG cells (A) or C57MG cell lines expressing Wnt-1 (B), WntCD8 (C), or WntCD4 (D,E) were plated at subconfluent densities. The medium was then changed to HB-CHO; cells were photographed 2 days later at a magnification of $200\times$ (see Materials and methods). A C57MG cell line infected with a recombinant virus construct intended to express an unrelated Wnt-1 fusion protein, but which did not produce detectable levels of it, is shown for comparison (control, F). The immunoblot at bottom displays the relative amounts of Wnt proteins in the indicated cell lines.

formed into elongated, refractile, fibroblast-like cells (B). C57MG cells making WntCD8 (C), WntCD4, or WntCD8 (data not shown) showed an intermediate type of morphology, implying that the chimeric proteins are active but less potent than wild-type Wnt-1. A similar morphology was seen with C57MG cells expressing sWntCD8 (not shown). This indicates that the reduction in activity seen with WntCD8 is the result of an effect of the carboxy-terminal CD8 portion of the chimera rather than a direct consequence of the attachment of Wnt-1 to the cell surface. Alternatively, the attenuation of activity may result, in part, from a reduction in efficiency of transport of WntCD8 to the cell surface relative to the efficiency of secretion of Wnt-1 protein, because sWntCD8 is secreted less efficiently than Wnt-1 (see Fig. 2). The partial transformation induced by these chimeras was not observed with WntCD8 C369W, a chimera containing an inactivating mutation.

C57MG cells expressing the short WntCD4 construct showed a very subtle but reproducible change in morphology; two independent examples are shown (Fig. S5D,E). The cells were not markedly elongated, but they extended processes and were slightly more refractile than controls. Only cell lines in which the fusion protein could be detected by immunoblotting (Fig. 5, bottom) showed the morphological changes. A C57MG cell line that failed to express a different Wnt fusion gene showed no alteration in phenotype and provided an independent control (F).

Wnt-1/transmembrane chimeras can transform C57MG cells by a paracrine mechanism

The activity of the chimeras in C57MG cells could be the result of the interaction of the Wnt-1 portion with its receptor in the ER of the expressing cells, on the surface of expressing cells, on the surface of adjacent cells, or a combination of these possibilities. To determine whether the chimeric proteins could also transform C57MG cells strictly by a paracrine route, Rat B1a cell lines expressing the chimeric proteins were cocultured with C57MG cells in an approximately equal ratio. The findings (summarized in Table 1 and illustrated in Fig. 6) were very similar to those obtained by introducing the chimeric constructs directly into C57MG cells (see above). Although coculture of C57MG and parental Rat B1a cells did not result in the transformation of the C57MG cells (Fig. 6A), mixing with Rat B1a cells producing Wnt-1 led to a change in morphology indistinguishable from that seen when Wnt-1 was expressed directly in the C57MG cells (cf. Figs. 5B and 6B; the donor cells are not visible because of overgrowth of the C57MG monolayer). Coculture of C57MG cells with Rat B1a clones producing WntCD8 (Fig. 6C), WntCD8, sWntCD8, or WntCD4 (data not shown) resulted in an intermediate transformation, which was not seen with clones making the WntCD8 C369W control chimera (D). The short WntCD4 protein, however, did not show paracrine transforming activity (not shown). Possible explanations for this observation are presented in the Discussion.

Activity of Wnt-1/transmembrane chimeras in Xenopus embryos

We then asked whether the Wnt-1 fusion proteins tethered to the cell surface are able to induce a new dorsal axis in X. laevis embryos. RNAs encoding Wnt-1 or the various chimeras were injected into embryos at the two-
Activity of Wnt-1 as a transmembrane protein

Wnt-1 is active when it is produced as a transmembrane protein. The WntCD4 and WntCD8 chimeric proteins were able to elicit partial morphological transformation in C57MG cells either by direct expression or by a paracrine route after coculture with donor RatBla cells producing the chimeras. Paracrine transformation was not observed with a chimeric protein lacking the extracellular domain of CD4, suggesting the need for a spacer between Wnt-1 and the transmembrane domain (see below). The transmembrane Wnt-1 chimeric proteins were also able to induce a new dorsal axis in X. laevis embryos. Once again, there was a requirement for a spacer domain. The reduction in activity observed when Wnt-1 is fused to a transmembrane protein is the result of the addition of new sequences to the carboxyl terminus, because a secreted chimera containing the extracellular domain of CD8 was similarly attenuated. This effect of adding extra amino acids to the carboxyl terminus of Wnt-1 has also been observed in at least one other case, after addition of an influenza hemagglutinin epitope tag [J. Kitajewski and H.E. Varmus, unpubl.]

The WntΔCD4 chimera has no paracrine activity but has partial transforming activity when expressed directly in C57 cells. This observation could be explained by a requirement for a spacer between the transmembrane and Wnt-1 domains to reach the receptor on the adjacent C57MG cells. Autocrine transformation would then be a result of Wnt-1 binding to its receptor in the ER or on the surface of expressing cells, which presumably does not require the spacer domain. Alternatively, the conformation of Wnt-1 could be affected differently by the carboxy-terminal sequences of this chimera in the extracellular environment; hence, it might retain activity only in the ER. Another possible explanation is that paracrine

Discussion

The results presented in this report demonstrate that

| Table 2. Activity of Wnt-1/transmembrane chimeras in Xenopus embryos |
|------------------|-------------------|
| RNA              | Percentage of injected embryos showing a secondary axis |
|                  | 10 pg             | 50 pg             | 250 pg            |
| Wnt-1            | 96% [71]          | 100% [18]         |                   |
| WntΔCD4         |                   | 0% [5]            |                   |
| WntCD4          | 52 (25)           | 87 (23)           |                   |
| WntCD8          | 28 (36)           | 87 (23)           |                   |
| WntCD8ɛ         | 57 (92)           |                   |                   |
| WntCD8ɛ-C369W   | 0 (54)            |                   |                   |
| sWntCD8         | 38 (79)           | 55 (31)           |                   |
| CD8ɛ            | 0 (20)            |                   |                   |

*X. laevis* embryos were injected with the indicated dose of RNA and allowed to develop to the tailbud stage, when the number of embryos showing signs of dorsal axis duplication was determined. No duplications were observed following injection of water only (see Fig. 7).

More drastic dorsalizing effects, in addition to axis duplication, are seen at these doses of Wnt-1 RNA.

Higher doses (0.5-1 ng) have also been tested, still with no effect.
transformation by WntΔCD4 requires a much higher level of cell surface expression. Because this protein also lacks activity in the Xenopus assay, it is tempting to speculate that axis duplication depends on paracrine signaling. One testable prediction of this hypothesis is that retention of Wnt-1 in the ER of expressing cells should abrogate its paracrine and axis-inducing activity. Previously, we have attempted to do this by attaching the ER retention signal KDEL [Pelham 1990] to the carboxyl terminus of Wnt-1. However, the Wnt-1/KDEL protein was not retained and was as active as wild-type Wnt-1 (J. Mason, J. Kitajewski, and H.E. Varmus, unpubl.), implying that it adopts a conformation that prevents recognition of the KDEL motif by its receptor in the ER.

The WntCD4 and WntCD8 chimeras were expressed intact in transfected cells and in Xenopus oocytes, indicating that their activity is not attributable to a diffusible cleavage product that migrates away from expressing cells. However, we cannot rule out the remote possibility that a very low level of cleavage does occur, generating an undetectable amount of free Wnt-1 protein. We consider this possibility unlikely, as extended exposure of autoradiographs, such as that shown in Figure 2, did not reveal such cleavage products in the medium, even when we would have detected levels far below the levels of Wnt-1 protein and at least 40-fold lower than those of sWntCD8. It is formally possible, however, that undetected amounts of cleavage products are above a threshold required for activity. To fully explain our results in this way, it is also necessary to postulate that the cleavage products would have a much higher specific activity than the intact chimeras. This scenario is extremely unlikely, as all alterations made to the Wnt-1 protein so far have either not affected its activity or attenuated it (McMahon and Moon 1989; Mason et al. 1992, N.T. Parkin, J. Kitajewski, and H.E. Varmus, unpubl.); a version of Wnt-1 with greater than wild-type activity has never been observed.

Wnt-1 protein has been postulated to be associated with both the cell surface [Papkoff and Schryver 1990] and the ECM [Bradley and Brown 1990]. Our immuno-
fluorescence analysis confirms that this is the case, at least for RatB1a cells [Fig. 4] or QT6 cells (N.T. Parkin and H.E. Varmus, unpubl.) grown on glass coverslips. The activity of Wnt-1, when it is tethered to the cell surface by a linked transmembrane domain, demonstrates that cell surface-associated Wnt-1 is active. To determine whether ECM-bound Wnt-1 is active, Wnt-1-producing cells were removed from the matrix by treatment with EDTA. This treatment leaves most, if not all, of the Wnt-1 protein on the ECM on the dish, as determined biochemically [Bradley and Brown 1990] or by immunofluorescence (N.T. Parkin and H.E. Varmus, unpubl.). C57MG cells were then plated on this ECM; no changes in morphology were induced, suggesting that ECM-bound Wnt-1 may not be active (N.T. Parkin, J. Kitajewski, and H.E. Varmus, unpubl.; A.M.C. Brown, pers. comm.). However, several other models involving a requirement for ECM binding cannot be eliminated, such as the need for transient matrix association during presentation to the Wnt receptor, or for manifestations of Wnt-1 activity that are not tested by the C57 transformation assay.

Several other growth factors are active in the form of transmembrane proteins. The Drosophila developmental regulators encoded by Delta [Rebay et al. 1991] and bride of sevenless (boss) [Krämer et al. 1991] appear to be expressed solely as transmembrane proteins. Interestingly, in the case of boss, it has been demonstrated that the entire transmembrane protein is internalized by sevenless-expressing cells (Cagan et al. 1992). A similar mechanism may be involved in signaling by the Wnt-1/transmembrane chimeras. Other factors, including epidermal growth factor (EGF) [Mroczkowski et al. 1988], transforming growth factor-α [TGF-α] [Brincah et al. 1987], tumor necrosis factor [TNF] [Kriegler et al. 1988], colony-stimulating factor 1 [Rettenmier and Roussel 1989], and neu differentiation factor [Wen et al. 1987], are synthesized as transmembrane precursors that are subsequently processed to yield a soluble form of the factor. The intact precursors for EGF [Mroczkowski et al. 1989] and TGF-α [Brachmann et al. 1989, Wong et al. 1989] are active, as are engineered forms of both TNF and EGF, which are surface bound and not cleaved [Perez et al. 1990; Dobashi and Stern 1991]. In these cases, intracellular signaling occurs despite the lack of large spacer domains separating the presumed receptor-binding epitopes from the membrane, which is required for paracrine activity of WntCD4. The c-kit ligand, Steel, is also made as a transmembrane precursor [Flanagan and Leder 1990]; mutations that prevent expression of this precursor, but still allow production of active, soluble ligand, induce severe developmental defects, suggesting that the membrane-bound form of Steel has unique functions [Brannan et al. 1991; Flanagan et al. 1991].

Thus, there is a large group of growth factors whose range of action can be limited by expression as a transmembrane protein. Still others are bound to ECM components [Rathjen et al. 1990]. The extent of diffusion of Wnt-1 in vivo is probably restricted by its association with the cell surface and/or the ECM. This is presumably an important mechanism for restricting the effects of Wnt-1 to a very limited set of responsive cells. The studies presented in this report show that production of Wnt-1 as chimeric transmembrane proteins does not impair its activity when compared with a secreted fusion protein, and thus membrane-bound Wnt-1 may mimic the normal range of action of Wnt-1 in vivo. One way to establish that cell-associated Wnt-1 protein is sufficient for biological function would be to rescue the mutant phenotype of Wnt-1-deficient mice by appropriate expression of the Wnt-1/transmembrane chimeras in vivo.

The surface expression and activity of WntCD4 and WntCD8f suggest that it may be possible to utilize these chimeras to seek Wnt-1-binding proteins, possibly including its receptor. WntCD8f was initially constructed with this possibility in mind, as it has been shown that binding of antibodies to CD8 at the surface of T cells activates all of the signaling pathways involved in the T-cell activation response, including transcription of the interleukin-2 [IL-2] gene [Irving and Weiss 1991]. In this way, expression of the WntCD8f chimera in T cells, followed by binding by anti-Wnt-1 antibody or by the Wnt receptor, might induce IL-2 secretion. Similarly, because the cytoplasmic domain of CD4 is sufficient for lck association [Shaw et al. 1989], the activity of this kinase might also be used to assay for functional interactions with the Wnt-1 domain. In QT6 cells cotransfected with lck and WntCD4 expression constructs, WntCD4 is able to associate with lck [N. Parkin and H.E. Varmus, unpubl.]. This approach could conceivably be used to clone the Wnt receptor cDNA from an expression library.

Materials and methods

Construction of Wnt-1/transmembrane chimeras

WntCD8f. An AflII site was introduced just upstream of the predicted signal peptide cleavage site of CD8f [Irving and Weiss 1991] by site-directed mutagenesis [Kunkel et al. 1987] in a Bluescript plasmid [pBSKS]+, Stratagene]. The mutagenic oligonucleotide [5'-C CCA GCA TGG TTA AGC CAG-3'] to give WntCD8f. Amino acid 369 of Wnt-1 was mutated to cysteine by site-directed mutagenesis. The mutagenic oligonucleotide [5'-G CAC GAG TGG TTA AGC CAG-3'] to give WntCD8f. Amino acid 369 of Wnt-1 was mutated to cysteine by site-directed mutagenesis. The mutagenic oligonucleotide [5'-G CAC GAG TGG TTA AGC CAG-3'] to give WntCD8f. Amino acid 369 of Wnt-1 was mutated to cysteine by site-directed mutagenesis.

WntCD8. An EagI fragment from pBS/WntCD8f, which contains the Wnt-1 cDNA and a portion of the CD8 cDNA, was inserted into a pBS plasmid containing the human CD8a cDNA [pBS/T8F1.1, obtained from the Littman laboratory, UCSF] digested previously with EagI to remove the 5' end of the CD8 cDNA. WntCD4. An AflII site was introduced at the predicted signal peptide cleavage site of human CD4 in pBS/CD4 [a gift of Ned Landau, UCSF]. A mutagenic oligonucleotide: 5'-C CCA GCA

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**Activity of Wnt-1 as a transmembrane protein**
**GCT TTA AGG GGA AAC AAA GTG G-3'**. This site was used to fuse the CD4 cDNA to the Wnt-1/A Jury cDNA to make WntCD4.

WntACD4 A 351-bp NcoI fragment was isolated from the carboxyl terminus of the human CD4 cDNA, corresponding to 5 amino acids of the juxtamembrane extracellular domain, the membrane- spanning sequence, and the cytoplasmic domain. This fragment was ligated to the Wnt-1/A Jury cDNA that had been cleaved with A Jury and filled in with Klenow. The 5’ junction was sequenced to confirm that the correct reading frame was maintained.

sWntCD8 A stop codon was introduced by site-directed mutagenesis (oligonucleotide sequence: 5’-GCC TTA AGG GGA AAC AAA GTG G-3’) at the codon immediately preceding the start of the predicted transmembrane domain of WntCD8.

For expression in cultured cells, constructs were cloned into the retroviral vector pLHTCX ([L. Murphy and H.E. Varmus, unpublished]). This vector was derived from pLNCX [Miller and Rosman 1989] by replacing the neomycin-resistance-coding sequence with that for a hygromycin-resistance/thymidine kinase fusion protein [Lupton et al. 1991]. In this vector, the hygTK protein is expressed from the murine leukemia virus long terminal repeat (MLV LTR), whereas the inserted cDNAs are expressed from the cytomegalovirus (CMV) promoter.

For generation of RNAs for microinjection into Xenopus embryos, constructs were subcloned into the pSP64 T vector that contains the 5'- and 3'-untranslated regions (UTRs) of Xenopus β-globin [Krieg and Melton 1984]. To eliminate possible translational repression by the Wnt-1 UTR, the NcoI site present at the initiator AUG of the Wnt-1 cDNA was used to clone the chimeras into a pSP64T derivative with an appropriately positioned NcoI site (pSP36T; a gift of E. Amaya and M. Kirschner, University of California, San Francisco). Use of this vector proved to be essential for detection of the encoded proteins by immunoprecipitation after injection of the RNAs into Xenopus oocytes and metabolic labeling (data not shown).

For constructs that were subjected to site-directed mutagenesis, the portion of the clone transferred to the expression vectors was sequenced to ensure that inapparent second-site mutations were not responsible for changes in activity. Alternatively, two independent clones derived from the mutagenesis procedure were tested; no differences in activity were noticed in these cases.

**Cell culture and transfection**

C57MG cells [Vaidya et al. 1978] were grown in Dulbecco's modified Eagle (DME) medium H-21 supplemented with 10% fetal bovine serum (FBS) and 10 µg/ml of insulin. RatBla cells [Finney and Bishop 1993] were obtained from Robert Finney (Bishop laboratory, UCSF) and grown in DME H-21 with 7.5% calf serum and 2.5% FBS. The PA317 retrovirus packaging cell line [Miller and Buttimore 1986] was grown in DME H-21 with 10% FBS. All cell lines were grown at 37°C in 5% CO₂.

Recombinant virus was generated by transiently transfecting PA317 cells with pLHTC constructs by calcium phosphate coprecipitation (Sambrook et al. 1989) and harvesting medium over a 16-hr period 2 days later. C57MG or RatBla cells were infected with viral supernatants, in the presence of 8 µg/ml of polybrene, for 2–3 hr. The next day, cells were split 1:5 into medium containing hygromycin B at 250 µg/ml. Colonies were usually visible 6–8 days later. Individual colonies of RatBla infectants were picked and screened for Wnt-1 expression by immunoblotting. C57MG colonies were pooled, and parallel plates were used for protein analysis by immunoblotting and for autocrine transformation assays.

**Protein analysis and morphology assays**

C57MG cell lines and clones of RatBla cells were screened for Wnt-1 protein expression by immunoblotting using chemiluminescent detection as described previously [Mason et al. 1992]. For pulse-chase analysis, subconfluent 60-mm dishes of RatBla cells were labeled with 1 ml of 0.4 mCi/ml [35S]cysteine [ICN] in DME [minus cysteine] for 1 hr at 37°C, and chased in 1 ml of DME with 10% FBS and 1 mM suramin [FBA Pharmaceuticals, CT] for 4 hr. The medium was removed and cleared in an Eppendorf microcentrifuge for 10 min before addition of anti-Wnt antibody. Cell lysates were prepared by washing cells three times in cold phosphate-buffered saline (PBS), lysing them on the dish by rocking in 1 ml of TNT buffer [20 mM Tris at pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 20 µg/ml of aprotinin] for 10 min at 4°C, and clearing as above. Cell lysates and medium were incubated for 3–16 hr at 4°C with 1 µl of anti-Wnt-1 monoclonal ascites fluid [anti-Wnt-1 peptide A; Brown et al. 1987], and immune complexes were precipitated with BSA-blocked protein A-Sepharose [Sigma]. The beads were washed three times in cold TNT buffer, boiled in SDS loading buffer, and electrophoresed on 10% polyacrylamide gels. The gel was fixed, soaked in Enhance (Amersham), dried and exposed to X-ray film (Hyperfilm, Amersham).

To best judge the morphology of the C57MG cell lines expressing Wnt-1 and the chimeras, the medium was removed just before the cells reached confluence and replaced with HB-CHO (Hana Biologics, Irvine CA), a defined medium that enhances differences in morphology by flattening out the parental C57MG cells [Mason et al. 1992]. For paracrine assays, ~5 × 10⁵ RatBla cells were plated together with an equal number of C57MG cells in 60-mm dishes; the following day, the medium was changed to HB-CHO. Cells were photographed 2 days later with Polaroid film and a Nikon TMS inverted microscope at a magnification of 200×.

**Flow cytometry**

RatBla cells were removed from 60-mm plates by treatment with enzyme-free cell dissociation buffer (GIBCO) as recommended by the vendor. Cells were resuspended in 100 µl of PBS containing 2% FBS (staining buffer) and incubated with 10 µl of fluorescein isothiocyanate-labeled Leu2a (anti-CD8) or phycoerythrin-labeled Leu3a (anti-CD4) (Becton-Dickinson) for 30–45 min on ice. The cells were washed twice in staining buffer, resuspended in staining buffer containing 1 µg/ml of propidium iodide, filtered through a 35-µm nylon mesh, and analyzed in a fluorescence-activated cell scanner equipped with Lyss II software (Becton-Dickinson). Only viable cells (those that did not take up propidium iodide) were included in the analysis.

**Immunofluorescence**

Cells were plated on coverslips that had been precoated with fibronectin (covered in 100 µl of 0.1 mg/ml fibronectin, air-dried, and rehydrated) at low density. The next day, samples were washed twice in cold PBS containing 0.2% gelatin and 0.1% sodium azide (IF buffer), incubated with primary antibody [1:200 anti-Wnt-1 monoclonal ascites or 5 µg/ml of purified OKT8] in IF buffer for 20 min on ice, washed, incubated with secondary antibody (1:200 Texas Red-conjugated anti-mouse IgG; Accurate Antibodies), washed, fixed in 0.37% paraformaldehyde for 10 min on ice, and mounted in glycerol containing...
3% n-propyl gallate. Samples were examined using a Leitz Orthoplan 2 inverted microscope and photographed with Tri-X black and white film [at ASA 800] at a magnification of ~500x. Exposure times for samples stained with anti-Wnt-1 antibody were ~1–1.5 min.

**Xenopus embryo injections**

Capped RNAs were transcribed using SP6 RNA polymerase [Promega] and the cap analog m'GpppG [Pharmacia], as described by the supplier. Unincorporated nucleotides were removed on a G-50 column, and the RNAs were precipitated with ammonium acetate and ethanol, resuspended in DEPC-treated water, and quantitated spectrophotometrically. The integrity and concentration of the RNAs were confirmed by agarose gel electrophoresis and staining with ethidium bromide. Samples were then diluted so that the desired dose was contained in 25 nl.

*X. laevis* frogs were maintained, and embryos were obtained, fertilized, and injected essentially as described [Moon and Christian 1989]. Embryos were injected with 25 nl of RNA at the two-cell stage (~90 min after fertilization at 19°C), in the vegetal half of one blastomere, using an automatic injector. Neurula (stage 19–20) and tailbud (stage 28) stage embryos were examined for evidence of axial duplications and photographed. In cases where two independent clones of a particular construct were tested, no significant differences were observed and the results were combined for Table 2.

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