0026-895X/04/6501-45–55$20.00
MOLECULAR PHARMACOLOGY
Vol. 65, No. 1
2787/1118434
Mol Pharmacol 65:45–55, 2004
Printed in U.S.A.

Gene Profiling of Frizzled-1 and Frizzled-2 Signaling: Expression of G-Protein-Coupled Receptor Chimeras in Mouse F9 Teratocarcinoma Embryonal Cells

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Received July 3, 2003; accepted October 2, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Wnt-Frizzled signaling via heterotrimeric G-proteins controls various aspects of early development. Because Wnts may activate more than one Frizzled, understanding the downstream signaling mechanisms and target genes for Frizzled activation has been a challenge. We constructed functional, chimeric receptors with the ligand-binding and transmembrane segments from the β_2-adrenergic receptor and the cytoplasmic domains from either rat Frizzled-1 (Rfz1) or Frizzled-2 (Rfz2). Activation with β-agonist enables stimulation of only a single Frizzled pathway and profiling of genes targeted by this Frizzled-specific approach. Genes activated in mouse totipotent F9 teratocarcinoma cells solely by activation of the Rfz1 chimera include *Lefty1*, *STAM*, *JAB*, *Erk1*, *MyD118*, *Fcer Ig*, and *follistatin*, genes implicated in development. Stimulation of Rfz2 chimera, but not Rfz1, leads to activation of a smaller set of genes, including those for *REST/NRSF*, *Groucho*, *nucleophosmin*, and *Ubc4/5E2*. Activation of either Rfz1- or Rfz2-specific chimera leads, in these totipotent stem cells, to some differential activation of a common set of genes, including those for *Msx-1*, *Msx-2*, *CBP/P300-associated factor*, *ephrin A3*, and *Nip-3*. We demonstrate the utility of β_2-adrenergic receptor–Frizzled chimeras to provide the tools with which to activate and to probe Frizzled-specific downstream signaling to gene activation.

Wnts constitute a family of vertebrate genes encoding ligands essential to signaling in early development (Cadigan and Nusse, 1997). These secreted glycoproteins act via members of the *frizzled* gene family (Bhanot et al., 1996; Yang-Snyder et al., 1996; He et al., 1997) of G-protein-coupled receptors (GPCR) (Liu et al., 1999b, 2001; Ahumada et al., 2002). Signaling downstream of some Frizzled homologs in response to Wnt-1 or Wnt-8 leads to activation of the phosphoprotein Dishevelled, repressing the function of glycogen synthase kinase-3β (GSK-3) activity (Miller et al., 1999; Polakis, 2000). In the absence of Wnt, GSK-3 phosphorylates β-catenin, reducing its stability and abundance. Wnt signaling represses GSK-3 activity, and β-catenin accumulates in the nucleus, where it binds to members of the lymphoid-enhancer factor and T-cell factor (Lef/Tcf) classes of architectural high mobility group box transcription factors to activate genes involved in early development. Wnt-5A, in contrast, does not alter β-catenin levels in some cellular contexts, including mouse F9 cells; rather, it stimulates phosphodiesterase, decreases intracellular [cyclic GMP], increases intracellular [Ca^{2+}], and activates Ca^{2+}/calmodulin-dependent protein kinase II, calcineurin, and other downstream effectors (Kuhl et al., 2000a).

Frizzleds display seven hydrophobic, membrane-spanning segments, characteristic of members of the superfamily of GPCRs (Liu et al., 1999b, 2001; Malbon et al., 2001; Ahumada et al., 2002). Analysis of Wnt-sensitive pathways has been hindered by the lack of purified, biologically active Wnts and the need to make use of conditioned media from cells secreting Wnts. To overcome this obstacle, we constructed a chimeric receptor with the ligand-binding and transmembrane segments from the β2-adrenergic receptor (β_2AR) and the cytoplasmic domains from rat Frizzled-1 (Rfz1) and Frizzled-2 (Rfz2). Stimulation of mouse F9 clones expressing the Rfz1 chimera (β_2AR/Rfz1) with the β-agonist isoproterenol stimulates the canonical features of Wnts on β-catenin [e.g., stabilization of β-catenin and activation of β-catenin-sensi-

This work was supported by grant awards from the National Institutes of Health and the March of Dimes Birth Defect Foundation.

ABBREVIATIONS: GPCR, G-protein-coupled receptor; GSK-3, glycogen synthase kinase; β_2AR, β_2-adrenergic receptor; Rfz1, rat Frizzled-1; Rfz2, rat Frizzled-2; Lef/Tcf, lymphoid-enhancer factor/T-cell factor; PE, primitive endoderm; PCR, polymerase chain reaction; QPCR, real-time, quantitative polymerase chain reaction; ODN, oligodeoxynucleotide; TGF, transforming growth factor; REST/NRSF, repressor element RE-1 silencing transcription factor/neuronal restricted silencing factor; DN-Tcf4, dominant-negative form of T-cell lymphoid-enhancer factor 4; Dvl/Dsh, Dishevelled.

tive transcription factors (such as Lef/Tcf) and formation of primitive endoderm (PE)] in the same manner stimulated in cells expressing the Rfz1 by use of conditioned media containing Wnt-8 (Liu et al., 2001).

Stimulation of the β_2AR/Rfz2 chimera, in contrast to the stimulation of the Rfz1 chimera, results in increased intracellular [Ca^{2+}] and decreased intracellular concentrations of cyclic GMP, in the same manner stimulated in cells expressing the Rfz2 by use of conditioned media containing Wnt-5A (Ahumada et al., 2002). Stimulation of the Rfz1 chimera does not lead to changes in intracellular levels of Ca^{2+} or cyclic GMP, whereas stimulation of the Rfz2 does not lead to appreciable stabilization of β-catenin and activation of the Lef/Tcf-mediated transcription (Liu et al., 1999b; Kuhl et al., 2000b; Wang and Malbon, 2003). Thus, the ability of each chimera to stimulate downstream signaling mimics with high fidelity the effects of the Wnts mediated through authentic, wild-type Frizzled-1 or Frizzled-2. Having validated the use of the chimeric receptors and avoided the complications of using media conditioned with other hormones, growth factors, etc., we were well positioned to perform gene profiling to address fundamental questions in Wnt-Frizzled signaling. Mammals display 18 or more separate genes for Wnts as well as for Frizzleds, and there exists overlap in the ability of several Wnts to activate more than one Frizzled. Making use of these novel Rfz1 and Rfz2 chimeras, however, enabled us to pose the question: what genes are activated in response to stimulation of a *single* specific Frizzled? We focus on activation of two well known members of the Frizzled family, making use of β_2AR/Rfz1 and β_2AR/Rfz2 chimera to specifically activate downstream pathways of gene activation of Frizzled-1 and Frizzled-2, respectively. Employing DNA microarrays, real-time PCR, and protein expression analyses, we scanned potential target genes in response to the activation of Frizzled-1 versus Frizzled-2 in totipotent, mouse F9 teratocarcinoma cells in culture. Uncomplicated by the use of conditioned media containing Wnts and other growth factors as well as the ability of purified Wnts to activate multiple Frizzleds, this is the first study of gene profiling after activation of a single Frizzled. We report on gene clusters that are activated only by stimulation of Rfz1 chimera, genes activated only by stimulation of Rfz2, and some genes regulated downstream of activation of either Rfz1 or Rfz2.

Materials and Methods

F9 Cell Culture and Stably Transfected Clones. The mouse F9 teratocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA), propagated, and stably transfected using LipofectAMINE (Invitrogen, Carlsbad, CA). The cells were transfected with pCDNA3 expression vector (Invitrogen) alone (empty vector) or pCDNA3 harboring either β_2AR/Rfz1 (hereafter "Rfz1 chimera") or β_2AR/Rfz2 (hereafter "Rfz2 chimera) (Liu et al., 2001). Some 10 to 20 independent clones that were resistant to G-418 were propagated in each transfection. The expression of the mRNA for each of the target proteins was measured indirectly via RT-PCR. The clones displaying the highest level of expression of mRNA for the chimera were employed for the studies. The levels of receptor expressed for the stably transfected clones was determined by equilibrium binding assays of the β_2AR binding domain of the chimera, using the radioligand iodocyanopindolol and intact cells (Liu et al., 2001). The mean values of receptor expression were as follows: Rfz1 chimera, 10.6 $\pm$ 2.1 fmol/10^5 cells, and Rfz2 chimera, 20.6 $\pm$ 4.4 fmol/10^5 cells (Liu et al., 1999a,b). F9 clones stably expressing a Frizzled chimera were induced to PE by the addition of 10 μM isoproterenol (Sigma, St. Louis, MO) and continued growth for 4 to 5 days. This concentration of isoproterenol was shown to yield maximal activation of Frizzled-1 and Frizzled-2 chimera in downstream read-outs (Liu et al., 1999a,b). In some cases, F9 cells were transiently transfected with the dominant-negative interfering mutant form of Tcf4 (DN-Tcf4) as detailed elsewhere (Kolligs et al., 1999; Liu et al., 2002). Activation of Rfz1, Rfz2, or their respective chimeras does not influence intracellular concentrations of cyclic AMP. Incubation media was supplemented, however, with the protein kinase A inhibitor KT-5720 (1 μM) to suppress any signal from the small complement of endogenous β_2-adrenergic receptors expressed by F9 cells.

Affymetrix Oligonucleotide Array. Total cellular RNA was isolated with RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) from either β_2AR/RFz-1 or β_2AR/Rfz2 stably transfected F-9 cells at times 0, 3.5, 6, 16, and 45 h after 10 μM isoproterenol treatment as described previously (Liu et al., 2001). Subsequent RNA processing procedures and Affymetrix Murine Genome Array U74Av2 application followed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA) by the University DNA Microarray Facility (Stony Brook, NY). The data sheet for Affymetrix Murine Genome Array for the U74Av2 chips can be found at http://www.affymetrix.com/products/arrays/specific/mgu74.affx. Data were analyzed using Affymetrix Genechip and GeneSpring software. High quality reproducible gene profile was facilitated by the analysis of time-courses from cells in which either Rfz1 or Rfz2 chimera were stimulated and then followed over time. The time points selected for analysis in the time-courses were chosen to make optimal the detection of the genes regulated by either of the Frizzled pathways. The entire gene profiling time course was performed twice using different clones on separate occasions to create a list of likely gene targets for validation by real time quantitative polymerase chain reaction (QPCR). The analysis of the data was performed at the University DNA Microarray Center (http://www.osa.sunysb.edu/udmf/) and with the help of the Bioinformatics Core Facility (http://www.osa.sunysb.edu/bioinformatics/index.html) at SUNY-Stony Brook. Software employed in the analyses included the GeneSuite software of Affymetrix and the GeneSpring software.

Real-Time Quantitative PCR. Real-time quantitative reverse transcription-PCR amplification was performed on the DNA Engine Opticon continuous fluorescence detection system (MJ Research Inc., Boston, MA) by monitoring the increase of fluorescence produced by the incorporation of SYBR green to the double-stranded DNA. The melting curve analysis was performed at the end of each PCR reaction to ensure that there was only one specific product. The PCR primers were designed with Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The first-strand cDNA was synthesized from 5 μg of total RNA using random hexamers with Superscript II reverse transcriptase (Invitrogen) and diluted to 0.3 ml of water. For a 20-μl PCR, 8 μl of cDNA template was mixed with forward and reverse primers (6.25 pmol of each primer for each reaction) and 2$\times$ SYBR Green PCR Master Mix (QIAGEN, Valencia, CA). The reaction was run at the setting program (40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min). Each cDNA sample was performed in duplicate gene-specific PCR. The gene specific primers were detailed in Table 1. For quantification purpose, the relative percentage changes were normalized in each sample against the expression of a gene that is not regulated by development, cyclophilin A. Typically, the target genes identified by Affymetrix-based gene profiling of the time-courses were then subjected to validation by employing real-time PCR analysis of three separate, detailed time-courses of wild-type F9 cells (or cells harboring empty expression vector), as well as of those clones expressing either the Rfz1 or the Rfz2 chimera. The graphs shown represent the mean values of the data from the three individual real-time PCR analyses. Variance and statistical significance were analyzed using SRS PRISMA software

TABLE 1
Primer oligomers employed for QPCR. The forward and reverse primers employed for QPCR of these genes of interest are described below.

Gene	Accession No.	Forward Primer	Reverse Primer
Cyclophilin A	NM 008907	5′-AGCACTGGAGAGAAAGGATTTG-3′	5′CACAATGTTCATGCCTTCTTTC-3′
Msx-1	NM 010835	5′-TACCACCTGGTCCCTTCTCTTA-3′	5′-CAATTTCTCTGTGCAAGTCCTG-3′
Msx-2	XM 122553	5′-GAACGCAGGGTCAAGGTCTC-3′	5′-CTCCTGTATCCACGGTGCTC-3′
CBP/P300-associated factor	NM 020005	5′-ACCACATAAAGCACGAGATCCT-3′	5′-AAACACGAAAGTCCAGGGTAGA-3′
Follistatin	NM 008046	5′-CTTTGTGGAAATGATGGAGTGA-3′	5′-ATGGAGTTGCAAGATCCAGAAT-3′
Ubc4/5E2	NM 009455	5′-TTCAAGCCTCCAAAGGTTACAT-3′	5′-AGCGTATCTCTTGGTCCACTGT-3′
REST/NRSF	XM 132128	5′-CAATGGATGTCTCCAAAGTGAA-3′	5′-TTACAGGGCTTTTCTGGATTGT-3′
AA968123		5′-ACCAATCAAAGCTCTACCACCT-3′	5′-GAGCCAGAGCTATGAAGGAAGA-3′
Nip-3	GI 2773345	5′-CAGCATGAATCTGGACGAAGTA-3′	5′-TAACGCTTGTGTTTCTCATGCT-3′
MyD118	X54149	5′-AGAGGAGGATGATATCGCTCTG-3′	5′-TAGATGTTTGGAGTGGGTCTCA-3′
Lefty1	D 83921	5′-CACTGCCCTTATCGATTCTAGG-3′	5′-CAATTGCCTTGAGCTCCATAGT-3′
STAM	MMU 43900	5′-TCATTGATGAAAAGCTGGAAGA-3′	5′ACTGCTCCTTGGCTGATAGAAG-3′
JAB	AB 000677	5′-GACACTCACTTCCGCACCTT-3′	5′-GAAAAGGCAGTCGAAGGTCTC-3′
Fcer Ig	NM 010185	5′-AGCTCTGCTATATCCTGGATGC-3′	5′-TGGTTTTTCATGCTTCAGAGTC-3′
Ephrin A3	U 92885	5′-CAGGTGAACGTGAACGACTATC-3′	5′-AATTCATAGCCCAGCGAGAAG-3′
Nucleophosmin	NM_008722	5′-CTTACGGTTGAAGTGTGGTTCA-3′	5′-TCATCATCATCCTCATCATCCT-3′
TLE/Groucho	NM_011599	5′-AAGATTCAGGCCTGTGTGACTT-3′	5′-CCTCTCTAATTGCCAGAGTGCT-3′
Erk1	XM_133759	5′-TACATTGTTCAGGACCTCATGG-3′	5′-TGTAGCCCTTGGAATTAAGCAT-3′

(Lion Biosciences, Cambridge, MS). The variance observed among the three separate real-time PCR determinations routinely was less than 20% of the mean values. The S.E.M. bars are not included on the graphs because of the dense, data-rich nature of the time-courses displayed for the real-time PCR analyses. The real-time PCR proved invaluable in the validation of the gene chip data. As will be noted, some genes highlighted in the DNA microarray were not validated as targets for Rfz1 or Rfz2-mediated pathways and consequently were deleted from further studies at the protein and function levels.

Antisense Oligodeoxynucleotides and Transfection Studies. Phosphorothioate-modified, high-performance liquid chromatography-purified oligodeoxynucleotides (ODNs) were obtained from QIAGEN Operon (Alameda, CA). The sequences were detailed in Table 2. The F9 clones expressing the β_2AR/Rfz1 chimera were propagated on 12- or 24-well plates and allowed to attach overnight. The clones were treated with antisense or mis-sense ODNs 48 to 72 h in advance of challenge with 10 μM isoproterenol.

Western Blot Analysis. For immunoblot analysis, the following polyclonal antibodies were used: rabbit polyclonal anti-ephrin A3, goat polyclonal anti-Nip3, goat polyclonal anti-JAB, rabbit polyclonal anti-Msx-1, rabbit polyclonal anti-Lefty, goat polyclonal anti-STAM, rabbit polyclonal anti-Msx-2, goat polyclonal anti-REST/NRSF, and rabbit polyclonal anti-Erk 1 (Santa Cruz Biochemicals, Santa Cruz, CA). Rabbit polyclonal MyD118 was kindly provided by Dr. Dan Liebermann (Temple University, Philadelphia, PA). Cell extracts were denatured, separated by SDS-PAGE, and electroblotted onto nitrocellulose membranes. After blocking, the blots were incubated with the primary antibody for 2 h, washed, incubated with secondary, horseradish peroxidase-coupled antibody of choice for 1 h, washed again, and then visualized by enhanced chemiluminescence. Equal loading was demonstrated by reprobing the membrane with anti-β-tubulin monoclonal antibody (Sigma).

Results

Cellular RNA was isolated from F9 clones stably expressing either β_2AR/Rfz1 or β_2AR/Rfz2. Clones were stimulated with the β-agonist isoproterenol (10 μM) for 0 to 45 h and subjected to gene profiling (Table 3). Stimulation of clones expressing β_2AR/Rfz1 chimera with β-agonist provokes the formation of primitive endoderm. This PE formation is maximally induced with 10 μM β-agonist and specifically blocked by simultaneous treatment with the β-adrenergic antagonist propranolol (10 μM) (Liu et al., 2001). The gene chips included more than 6000 functionally characterized genes of the Mouse UniGene set (build 74) and more than 6000 expressed sequence tag clusters on the murine chip. The approach adopted was to perform DNA microarray from multiple clones in which either the Rfz1 or the Rfz2 pathway was activated and to study the gene profiles from detailed time-courses, rather than from a single time point. The initial times selected for sampling of gene activation were 0, 3.5, 6, 16, and 45 h after stimulation of clones expressing one or the other chimera. Displayed in Table 3 are the names of the regulated genes, their accession numbers, the maximal values of change (-fold increase), and the times at which peak change occurred for each gene. Fewer than 30 genes were activated at >1.5-fold over unstimulated clones, in response to activation of either Rfz1 or Rfz2 chimeras. Based upon the DNA microarray data, several of the target genes were found to be activated only by β_2AR/Rfz1 chimera (e.g., *Dvl-2*) or by β_2AR/Rfz2 chimera (e.g., *MmZw10*), whereas other genes (e.g., *Lefty1*, *MyD118*, and *STAM*) were activated by stimulation of either Rfz1 or Rfz2 chimera. We validated putative target genes using QPCR employing cellular RNA isolated from clones stimulated without or with β-agonist for 1 to 45 h (Table 4). Stimulation of either Frizzled chimera activated *Msx-1*, *Msx-2*, *CBP/P300-associated factor*, *ephrin A3*, and *Nip3* genes. *Follistatin*, *Lefty1*, and *STAM* were early targets of gene activation by Rfz1, whereas *REST/NRSF*, *Ubc4/5E2*, *nucleophosmin*, and *Groucho* were early targets for gene activation by Rfz2.

The results of QPCR confirmed most but not all (e.g., *Dishevelled-2* and *sFRP-1*) of the target genes identified by DNA microarray and extended our description of the temporal up-regulation of these RNAs (Fig. 1A). We grouped those genes whose expression was identified first by DNA microar-

TABLE 2
Oligonucleotide sequences employed to validate the role of candidate genes in signaling from Frizzleds to formation of primitive endoderm by mouse F9 teratocarcinoma cells
The sequences for both the antisense and the mis-sense ODNs are provided.

Gene	Antisense ODN	Missense ODN
Msx-1	AGCTCTGTCGGGACCTAGTA	AGCTCGCGTCGCTGATAGTA
STAM	ACAGGCGACGCACGGCTCCAC	ACAGGGACGGCGCACCTCCA
JAB	CCTACTCGAGGGGCCAGCTG	CCTACAGTTGACGTCAGCTG
Ephrin A3	CTGTGGACAAATGAAGTGGG	CTGTGAGTAGCGAATGTGGG
MyD118	CCACAGCGGACCCCCACGGA	CCACATACCGATGCTACGGA
Nip3	GAACCCAACTGCGACAGGCC	GAACCATGTGGTCGTAGGCC

TABLE 3

Transcriptional responses of mouse F9 teratocarcinoma cells to activation of Frizzled-1 or Frizzled-2: DNA microarray (partial list)

The products of the following genes were identified by gene profiling of mouse F9 teratocarcinoma cells expressing the rat Frizzled-1 (Rfz1) or the rat Frizzled-2 (Rfz2) chimera. Maximal change and time of response are shown.

Gene	Accession No.	ΔMax	Time	Description	Reference
			h		
Rfz1 chimera					
Dishevelled 2	U24160	2.94	3.5	Phosphoprotein intermediated in Wnt signaling	Capelluto et al.,2002
Lefty1	D83921	1.83	3.5	TGF-beta-related gene of early embryonic patterning	Kothapalli et al., 1997
SRPK1	AB012290	1.46	16	Small nuclear ribonucleoprotein specific kinase 1	Gui et al., 1994
Fcer Ig	AV012229	2.94	16	Receptor for IgA	Maliszewski et al., 1990
Dnal4	BAA24153	1.81	16	ATPase dynein light chain 4	Ogawa et al., 1996
Ephrin A3	U92885	1.91	16	Ligand for Eph receptor tyrosine kinase	Cerretti et al., 1996
BING-1	AF100956	3.06	16	Zinc finger protein	Herberg et al., 1998
JAB/SOCS1	Z47352	3.19	16	Janus kinase-binding protein	Endo et al., 1997; Starr et al., 1997a,b
Bip	P20029	4	16	Member of hsp 70 family of chaperones	Lee et al., 1999
Rap-2b	AI837726	4.19	16	Ras-related protein	Ohmstede et al., 1990
MyD118	P22339	9.93	16	Growth suppression and pro-apoptotic gene	Abdollahi et al., 1991
JIP1	AF003115	1.82	45	An inhibitor of c-Jun N-terminal kinase	Yasuda et al., 1999
Erk1	Z14249	1.83	45	Mitogen-activated protein kinase for cell proliferation	Charest et al., 1993
STAM	U43900	2.41	45	Signal-transducing adaptor molecule of Janus kinase	Takeshita et al., 1996
Nip3	AF041054	4.98	45	E1B 19K/Bcl-2-binding protein	Boyd et al., 1994
Rfz2 chimera					
sFRP-1	U88566	1.51	3.5	Soluble modulators of Wnt signaling	Roth et al., 2000
Lefty1	D83921	1.53	3.5	TGF-beta-related gene of early embryonic patterning	Kothapalli et al., 1997
Blast to hypothetical protein	AA968123	2.67	3.5	Unknown	
MmZW10	AF003951	3.12	3.5	Cell division protein involved in chromosomal segregation	Starr et al., 1997a,b
Erk5	AB019373	2.79	6	Mitogen-activated protein kinase for cell proliferation	Zhou et al., 1995
Gnai 2	AI841629	1.9	16	G-alpha inhibitory protein of cell signaling	Blatt et al., 1988
JIP1	AF003115	2.29	16	An inhibitor of c-Jun N-terminal kinase	Dickens et al., 1997
Erk1	Z14249	2.52	16	Mitogen-activated protein kinase for cell proliferation	Charest et al., 1993
STAM	U43900	3.05	16	Signal-transducing adaptor molecule of Janus kinase	Takeshita et al., 1996
MyD118	P22339	3.8	16	Growth suppression and pro-apoptotic gene	Abdollahi et al., 1991
Bip	P20029	3.88	16	Member of hsp 70 family of chaperones	Lee et al., 1999
P-cadherin	P10287	1.61	45	Ca^{2+}-dependent adhesion protein in development	Nose et al., 1986
Nip3	AF041054	4.3	45	E1B 19K/Bcl-2-binding protein	Boyd et al., 1994

TABLE 4

Gene profiling in response to activation of Frizzled chimera: QPCR validation of targets identified by DNA microarray analysis

Genes of interest regulated by activation of either Frizzled-1 or Frizzled-2 chimeras were first identified by gene profiling were subjected to QPCR over an extended time frame after activation of the chimera.

Gene	-Fold Increase	Time	-Fold Increase	Time	Description
	Δmax	*h*	Δmax	*h*	
Rfz1 chimera					
CBP/P300-associated factor	1.93	1	1.93	1	Nuclear transcriptional coactivator protein associated factor
Msx-2	1.99	1	1.99	1	Muscle segment homeobox containing gene in development
Follistatin	2.46	1	2.46	1	Activin-binding protein, activin antagonist
Lefty1	2.71	1	9.08	30	TGF-β–related gene of early embryonic patterning
STAM	1.74	1	2.57	45	Signal-transducing adaptor molecule of Janus kinase
JAB/SOCS1	2.69	4	2.69	4	Janus kinase-binding protein
Ephrin A3	2.13	4	2.13	4	Ligand for Eph receptor tyrosine kinase
Msx-1	2.28	4	2.28	4	Muscle segment homeobox containing gene in development
Fcer Ig	1.86	4	2.19	30	Receptor for IgA
Erk1	1.77	20	1.77	20	Mitogen-activated protein kinase for cell proliferation
Nip-3	2.05	20	2.05	20	E1B 19K/Bcl-2-binding protein
MyD118	2.00	20	2.36	45	Growth suppression and pro-apoptotic gene
Rfz-2 chimera					
Msx-2	2.69	1	2.69	1	Muscle segment homeobox containing gene in development
REST/NRSF	3.20	1	3.20	1	Zinc-finger gene-specific repressor element RE-1 silencing factor
Ubc4/5E2	1.81	2	1.81	2	Ubiquitin-conjugating enzyme homolog of yeast Ubc4
Nucleophosmin	1.55	2	1.55	2	Nucleolar phosphoprotein
Ephrin A3	1.45	2	1.70	4	Ligand for Eph receptor tyrosine kinase
TLE/Groucho	1.35	2	1.60	45	Co-repressor mediating negative transcriptional control
CBP/P300-associated factor	2.38	2	7.59	4	Nuclear transcriptional coactivator protein
Msx-1	2.33	10	4.73	45	Muscle segment homeobox containing gene in development
AA968123	2.17	20	2.17	20	Unknown
Nip-3	2.25	45	2.25	45	E1B 19K/Bcl-2-binding protein

ray and later confirmed independently by QPCR analysis of multiple, separate time-courses into clusters of genes activated by Rfz1 chimera only (Fig. 1A), by Rfz2 chimera only (Fig. 1D), and by both Rfz1 and Rfz2 chimeras (Fig. 1G). To facilitate data presentation, the traces are grouped temporally and displayed in one of three different colors. RNA for the activin-binding protein follistatin, the TGFβ-related Lefty1 gene product implicated in early embryonic patterning in vertebrates, and signal-transducing adaptor molecule of Janus kinase STAM RNAs increased 1- to 3-fold within 1 to 2 h of Rfz1 activation and declined thereafter within 4 h (Fig. 1A, red traces). Lefty1 and STAM RNA levels increased later; Lefty1 RNA reached peak levels (~9-fold over basal) by 30 h after stimulation of Rfz1 chimera. RNAs for Janus kinase-binding protein JAB (SOCS1), and IgA receptor Fcer Ig, in contrast, displayed a later onset at 2 to 5 h after Rfz1 stimulation, declining at the 5- to 10-h period, maintaining nearly 2-fold elevation to 30 h (Fig. 1A, green traces). The mitogen-activated protein kinase Erk 1 and growth suppressor/pro-apoptotic MyD118 RNAs, in contrast, peaked at 20 h after Rfz1 chimera stimulation (Fig. 1A, blue traces). Treating the clones expressing the Rfz chimera with the β-adrenergic antagonist propranolol (10 μM) blocked activation observed in response to isoproterenol of the following genes studied: *CBP/P300-associated factor*, *ephrin A3*, *Lefty1*, *msx2*, *MyD118*, and *Nip3*. Thus, we demonstrated clusters of genes that were being activated in response to activation of the Rfz1 pathway in these mouse F9 cells.

Several controls were employed throughout these studies. Studies with wild-type F9 cells were included to detect any gene expression not attributable to the activation of either the Rfz1 chimera (Fig. 1B) or the Rfz2 chimera (Fig. 1E). Activation of these target genes by wild-type F9 cells in response to isoproterenol was not observed. The inactivation of several of these genes noted in the wild-type cells in response to isoproterenol (Fig. 1, B and E) was sensitive to treatment with the antagonist propranolol (data not shown). The results from multiple experiments reveal no significant activation of the genes stimulated in clones expressing the Rfz1 chimera (Fig. 1A) in the similarly treated wild-type F9 cells (Fig. 1B). In addition, we performed QPCR of the Rfz1-sensitive genes in the clones expressing the Rfz2 chimera (Fig. 1C). Confirming the results from the DNA microarray (Table 3), the QPCR data revealed no significant activation of the Rfz1-sensitive genes after activation of the Rfz2 chimera (Fig. 1C). Thus, good agreement exists between the data from the time-courses analyzed by DNA microarray and independently from the studies employing QPCR.

Activation of Rfz2 chimera caused short-term stimulation of RNA levels of the zinc-finger gene-specific REST/NRSF; the Groucho corepressor that mediates negative transcriptional regulation in association with DNA-binding proteins in development; the ubiquitin-conjugating enzyme (E2) homologous to the yeast UBC4 Ubc4/5E2; and the abundant nucleolar phosphoprotein nucleophosmin genes (Fig. 1D, red traces) with RNA levels declining below basal within 20 to 30 h (Fig. 1D). The activation of the Wnt-Ca^{2+} pathway by the Rfz2 chimera in response to isoproterenol was sensitive to antagonism with 10 μM propranolol (Liu et al., 1999b, 2001; Ahumada et al., 2002). Likewise, treating these clones with propranolol blocked the activation of gene expression stimulated by isoproterenol (data not shown). Analysis of these Rfz2-sensitive genes in wild-type F9 cells revealed no significant activation of these target genes by isoproterenol (Fig. 1E). Similarly, analyses by QPCR of the genes activated by Rfz2 were performed in clones expressing the Rfz1 (Fig. 1F). The activation of Rfz1, which led to enhanced expression of one set of genes subjected to analysis by DNA microarray and QPCR (Table 3 and Fig. 1A), did not lead to any significant activation of the genes stimulated in response to activation of Rfz2 chimera (Fig. 1F).

Interestingly, RNA levels of some target genes for activa-

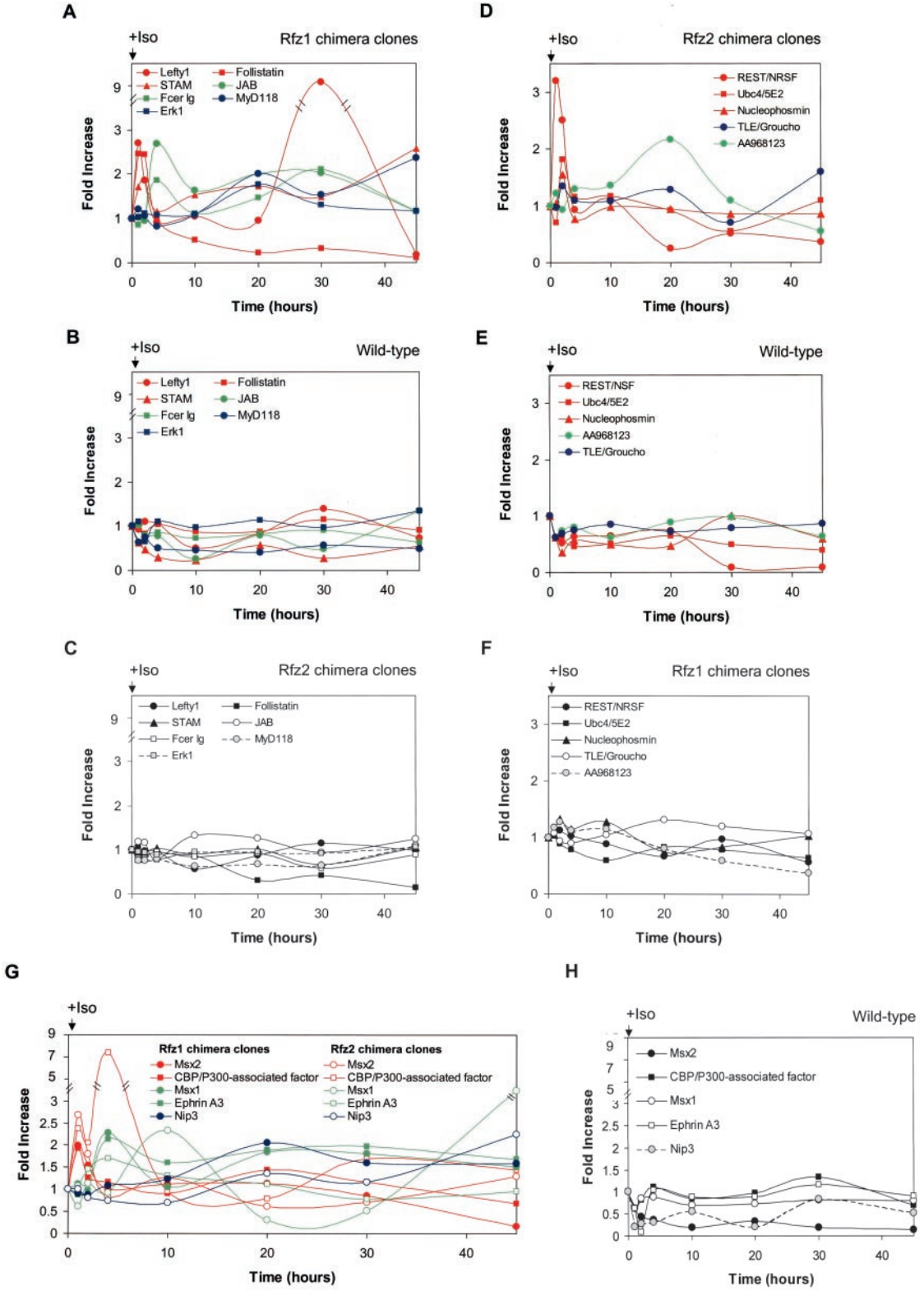

Fig. 1. QPCR analysis of target genes up-regulated by activation of rat Frizzled-1 or Frizzled-2 chimera in F9 embryonic teratocarcinoma cells. Wild-type F9 cells as well as individual stably transfected F9 clones expressing either Rfz1 or Rfz2 chimera were treated with or without the β-agonist isoproterenol (Iso, 10 μM) to activate Frizzled signaling. Total RNA was isolated at the times indicated in the activation profile and then reverse-transcribed to first-strand cDNA. Target genes for activation of Rfz1 (A–C), Rfz2 (D–F), or both (G and H) identified by DNA microarray were subjected to analysis by QPCR. All the regulated genes were tested in wild-type F9 cells (B, E, and H). As a control, Rfz1 target genes were examined in Rfz2 chimera clones (C) and vice versa (F). All values are displayed as means, each determined by the intensity ratio of the target gene to that of cyclophilin A. Minimally, two separate reverse transcriptions (RT) and a duplicate PCR for each reverse transcription were performed. The sequences for primers employed for QPCR are listed in Table 1.

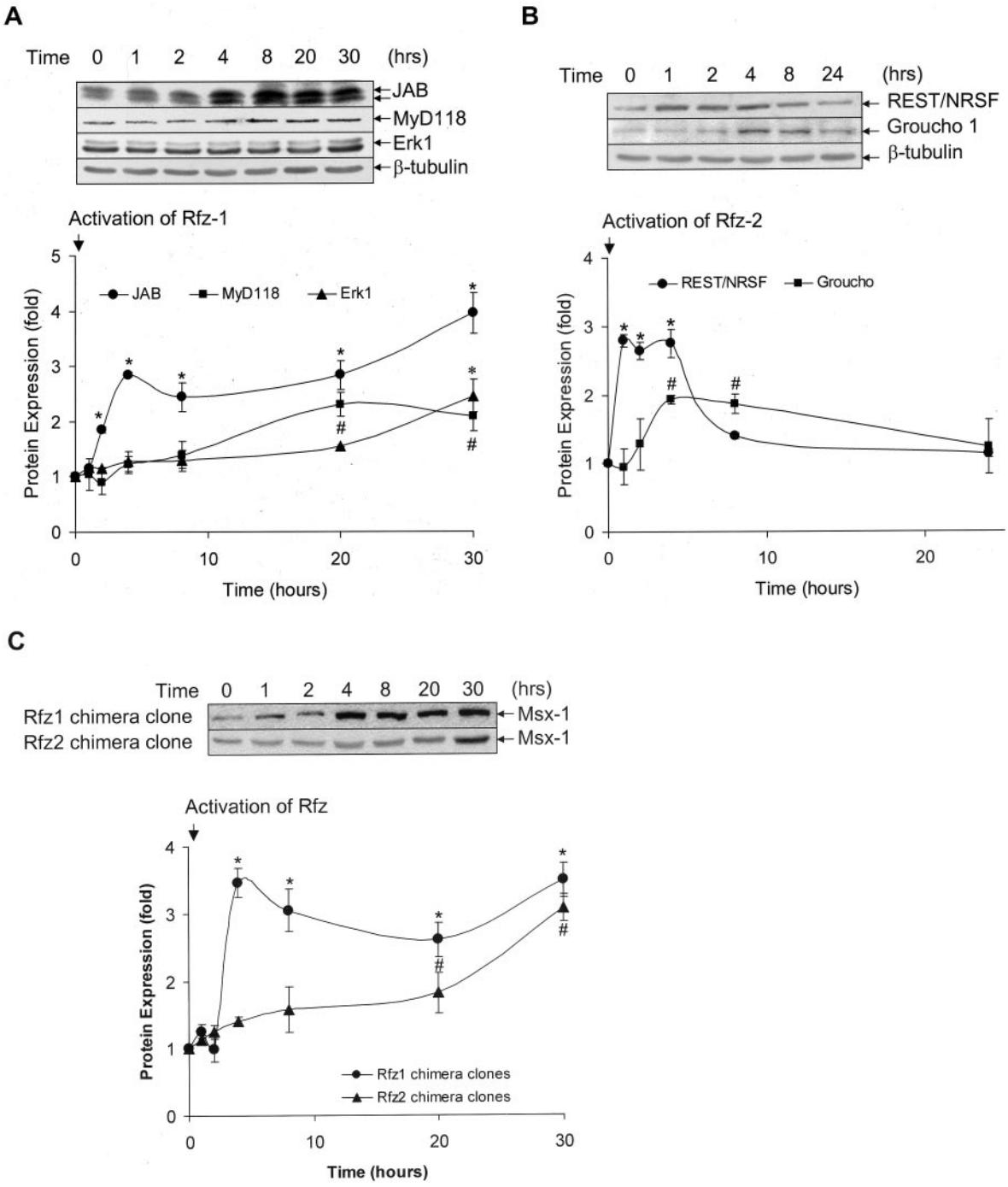

Fig. 2. Expression of target gene products after activation of either Rfz1 or Rfz2 chimera. Individual stably transfected F9 clones expressing either the Rfz1 or the Rfz2 chimera were treated with or without the β-agonist isoproterenol (Iso, 10 μM) to activate Frizzled signaling. Whole-cell lysates were prepared from the cells at the times indicated and then subjected to SDS-PAGE and immunoblotting. The blots were stained with the antibodies to the following gene products: Msx-1, JAB, MyD118, Erk1, REST/NRSF, and TLE/Groucho 1. The equivalence of protein loading was established by immunoblotting of β-tubulin. The results shown are representative of at least three separate experiments, each performed with different F9 clones. Gene products unique to stimulation of Rfz1 (A) or Rfz2 (B) chimera, or common to both (C) were monitored. *, #, $P < 0.05$, statistically significant difference from zero time point.

tion in response to either Rfz1 or Rfz2 stimulation (e.g., homeobox-containing genes in development Msx-1, Msx-2, CBP/P300-associated factor, the ephrin A3 ligand for the Eph tyrosine kinase, as well as E1B 19K/Bcl-2-binding protein Nip-3) displayed similarities in their time-courses (Fig. 1G). Although Rfz1 and Rfz2 display highly homologous (~85% identity) cytoplasmic sequences (Wang et al., 1996), they activate both unique and common sets of genes. Analysis in wild-type F9 cells of this subset of genes regulated in response to stimulation of either Rfz1 or Rfz2 revealed no significant gene activation (Fig. 1H).

Although QPCR studies enabled validation of a major subset of the Rfz-sensitive genes identified by DNA microarray and gene profiling, further validation of regulated expression

TABLE 5
Effects of antisense (suppression) versus missense (control) ODNs to target those genes implicated in the formation of primitive endoderm in response to activation of the rat Frizzled-1 in mouse F9 teratocarcinoma clones expressing the Rfz1 chimera

Target	ODN	PE Formation
JAB	Antisense	Blocked
	Mis-sense	No Effect
Nip-3	Antisense	Blocked
	Mis-sense	No Effect
Myd118	Antisense	Blocked
	Mis-sense	No Effect
Ephrin A3	Antisense	No Effect
	Mis-sense	No Effect
Msx-1	Antisense	Enhanced
	Mis-sense	No Effect
Stam	Antisense	Enhanced
	Mis-sense	No Effect

of the target proteins was crucial to detailed analysis of Rfz-stimulated genes. At the protein level, immunoblotting was employed to validate the QPCR data. Immunoblots of whole-cell extracts prepared from clones expressing the Rfz1 chimera activated with isoproterenol confirmed Rfz1-stimulated expression of key genes (Fig. 2). Activation of Rfz1 chimera led to increases in the several gene products: JAB, the coactivator of activator protein-1 transcription factor central to angiogenesis (Endo et al., 1997); and MyD118, a gene product involved in negative growth control, JNK suppression, and apoptosis (Abdollahi et al., 1991) (Fig. 2A). STAM, the signal-transducing adaptor in Janus tyrosine kinase-signal transducer and activator of transcription signaling and Lefty1, a TGFβ-related gene of early embryonic patterning were likewise up-regulated in response to activation of the Rfz1 chimera (not shown). The gene for the mitogen-activated protein kinase Erk 1 displayed activation and increased mRNA expression in response to stimulation of the Rfz1 chimera (Fig. 1A); however, its protein expression remained essentially unchanged for the first 20 h after stimulation (Fig. 2A).

A number of target genes, including *REST/NRSF*, *Groucho*, *nucleophosmin*, *Ubc4/5E2*, and *AA968123*, were activated by Rfz2, but not Rfz1. Several of these same genes were reported to be activated in the human teratocarcinoma cell line NCCIT treated with conditioned media prepared from mouse L-cells transfected with the expression vector for Wnt-3A (Willert et al., 2002). At the protein level, Rfz2 activation was found to provoke increased expression of a number of these targets, including the zinc-finger gene-specific repressor element REST/NRSF and the transcriptional corepressor of Wnt-Frizzled-1 signaling, Groucho 1 (Cavallo et al., 1998) (Fig. 2B). Several target genes demonstrated activation in response to either Rfz1 or Rfz2, especially members of the Msx homeobox gene products, Msx-1 and Msx-2, involved in inductive tissue interactions necessary for vertebrate organogenesis (Fig. 2C). Although activation of both Frizzleds activated Msx-1 expression, activation of the Rfz1 chimera stimulated an earlier, more robust response than did activation of the Rfz2 pathway. Members of this group of target genes (e.g., *Msx-1*, *Msx-2*, and *CBP/P300*-associated factor) also were activated by treatment of NCCIT cells with conditioned media containing Wnt-3A (Willert et al., 2002), thus providing good agreement from these DNA microarray analysis and recent studies performed with conditioned media containing authentic Wnt-3A. The genes for Nip3, a pro-apoptotic mitochondrial protein (Bruick, 2000) and for the ephrin A3 ligand for the Eph receptor tyrosine kinase were shown to be new members targeted by activation of both Rfz1 and Rfz2 chimera (Fig. 1G, Table 4).

We next analyzed the role of several target gene products in the ability of Rfz1 to stimulate early features of development in these F9 embryonic stem cells. The roles of Frizzled-1 activated target genes were investigated in the forma-

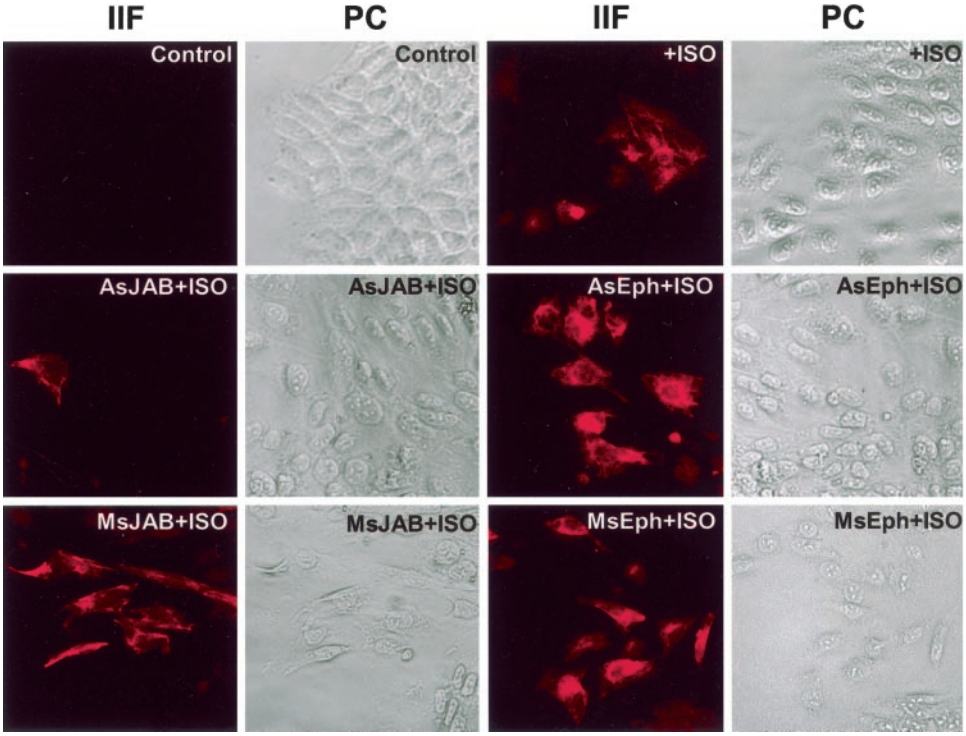

Fig. 3. Suppression of JAB, but not ephrin A3, by antisense oligodeoxynucleotides blocks formation of primitive endoderm in response to activation of Rfz1 chimera. Mouse F9 cells stably expressing the β_2AR/Rfz1 chimera were treated for 48 to 72 h with antisense ODNs (As) or missense ODNs (Ms, control) to suppress expression of either JAB or ephrin A3. Clones were treated with 10 μM isoproterenol (+ISO) and examined after 4 to 5 days for expression of cytokeratin endo A, the protein marker for PE formation, by staining with the TROMA-1 antibody. Immunoblots stained with the antibodies against the JAB and against ephrin A3 (not shown) revealed >50% suppression of the target protein after treatment with the antisense compared with mis-sense ODNs. Antisense ODN treatment to JAB (AsJAB), but not ephrin A3 (AsEph), decreased TROMA-1 staining by ~80%. Treatment of the clones with mis-sense ODNs to JAB (MsJAB) or to Eph (MsEph) had no effect on the ability of activation of Rfz1 to block the formation of PE (positive staining of the cytokeratin endo A) of the Rfz1 chimera-expressing clones. The results shown for the positive staining of the PE marker cytokeratin endo A are representative of at least three separate experiments.

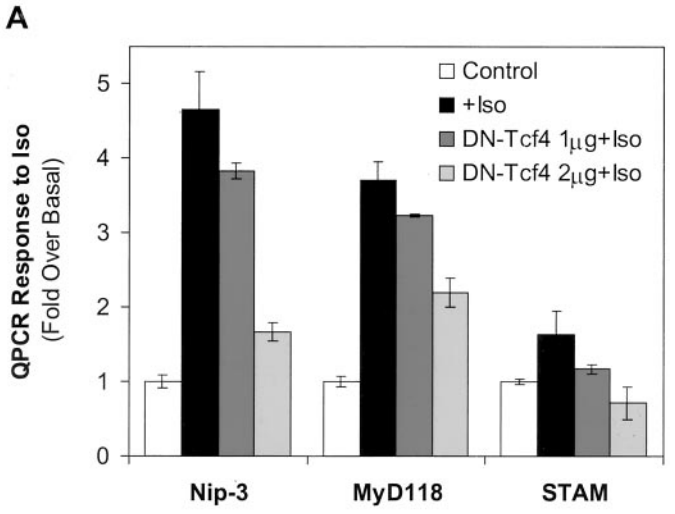

Fig. 4. Expression of dominant-negative Tcf4 blocks activation of *MyD118*, *Nip-3*, and *STAM* genes, but not *JAB* or *Msx-1* genes in response to activation of Rfz1 chimera. F9 clones stably transfected with expression vector for Rfz1 chimera were transiently transfected with either 1 or 2 μg of an expression vector harboring the dominant-negative Tcf4. The clones were activated with 10 μM isoproterenol (+Iso) for 24 h. The RNA levels for the targeted genes were analyzed by QPCR. Expression of DN-Tcf4 prevents the up-regulation of MyD118, Nip-3 and STAM by activation of Rfz-1 (A), but had no effect on RNA levels of JAB and Msx-1 (B). Data (mean ± S.E.M.) determined by the intensity ratio of the product of the target gene compared with that of cyclophilin A. Minimally, two separate reverse transcriptions and a duplicate PCR for each RT were performed.

tion of PE. F9 clones were treated with either antisense or missense (as a control) ODNs targeting the gene product of interest. The ability of the Rfz1 chimera to promote formation of primitive endoderm was determined in the clones made deficient in the target gene (Table 5, Fig. 3). Formation of PE was established by positive staining for the cytokeratin endoA, a hallmark protein for PE, with the TROMA-1 antibody (Liu et al., 2001). Treatment of the clones with ODN antisense, but not mis-sense, to JAB, Nip3, or MyD118 suppressed the expression of each (not shown) and blocked the ability of the activated Rfz1 to stimulate PE formation (Table 5, Fig. 3). These observations demonstrated an obligatory role of *JAB*, *Nip3*, and *MyD118* as key genes in the formation of PE in response to stimulation of the Frizzled-1 pathway. Interestingly, for two target genes, *Msx-1* and *STAM*, the treatment with antisense ODNs *increased* rather than *decreased* PE formation, suggesting functions of both *Msx-1* and *STAM* as *de*-differentiation factors in Frizzled-1 signaling.

Activation of the Lef/Tcf-dependent transcriptional response has been shown to be an essential early response for Rfz1 signaling in development (Cadigan and Nusse, 1997; Barker et al., 2000; Liu et al., 2001). We evaluated to what degree, if any, the β-catenin-sensitive Lef/Tcf transcriptional response to Rfz1 regulates the Rfz1-sensitive genes *MyD118*, *STAM*, *Msx-1*, and *JAB*. F9 clones stably expressing Rfz1 chimera were transiently transfected with an expression vector harboring the dominant-negative form (DN-Tcf4) of Tcf4 (Kolligs et al., 1999). The expression of DN-Tcf4 effectively blocks genes regulated by the Lef/Tcf-sensitive transcriptional pathway and has been shown previously to block Frizzled-1 stimulated PE formation in F9 embryonic stem cells (Liu et al., 2001). Using QPCR, we analyzed the level of mRNA for the targeted genes in clones transiently transfected with either 1 or 2 μg of DN-Tcf4 plasmid DNA (Fig. 4, A and B). Graded expression of DN-Tcf4 was found to produce a graded block of the Rfz1-stimulated activation of MyD118, Nip3, and STAM genes (Fig. 4A). The results justified a closer inspection of the Frizzled-1 sensitive genes for the presence of the DNA motif (A/T)(A/T)CAA(A/T)GG, conserved for Tcfs in vertebrates, *Drosophila melanogaster*, and the worm *Caenorhabditis elegans* (Roose and Clevers, 1999). This canonical motif was observed in the *follistatin* and *MyD118* genes, whereas the motif with one 5′ end substitution was found in early genes for *STAM* and *Lefty1*, as well as in *JAB* and *ephrin A3*. Interestingly, the genes for *CBP/P300-associated factor*, *Msx-1*, and *Msx-2* display the same DNA motif but were up-regulated by activation of either Rfz1 or Rfz2 chimera. Up-regulation of *JA*B and *Msx-1* by Frizzled-1, in contrast, was shown to be insensitive to DN-Tcf4 expression (Fig. 4B), revealing novel Lef/Tcf/β-catenin-*in*sensitive elements of Frizzled-1 signaling.

Discussion

Gene profiling of the Wnt-Frizzled signaling pathways is essential if we are to more fully understand how these pathways control cell polarity, embryonic induction, specificity of cell fate, and diseases, such as cancer, attributed to lesions in one or more of the Wnt-Frizzled pathways (Wang and Malbon, 2003). More than 20 Wnt ligands are expressed during mammalian development. The overarching goal of these studies was to dissect out Frizzled-specific pathways, using a strategy that obviates the ability of Wnts to activate multiple Frizzleds, a gene family that itself has more than 18 members in mammals. We made use of the chimeric GPCRs composed of the transmembrane and exofacial domains of the β_2AR to which Frizzled cytoplasmic domains were substituted, creating functional β_2AR/Rfz1 and β_2AR/Rfz2 chimeras. These unique studies provide the first insights into transcriptional responses to Frizzled-specific signaling GPCRs that mediate key aspects of early vertebrate development (Malbon et al., 2001). First and perhaps most importantly, we show both common as well as unique patterns of transcriptional responses after activation individually of Rfz1 and of Rfz2, highly homologous GPCRs (Bhanot et al., 1996). The transcription factors REST/NRSF, Groucho, and Ubc4/5E2 were shown previously to be up-regulated by treating NCCIT human embryonic carcinoma cells with conditioned media containing Wnt-3A (Willert et al., 2002) and by activation of Rfz2 chimera, but not by Rfz1 (current study). The use of conditioned media containing Wnt-3A to stimulate cells for gene profiling is fraught with limitations (Willert et al., 2002), but it is interesting to note the overlaps between the gene profiling reported by others and the data derived from the activation of a single Frizzled pathway. Most of the genes noted above have been linked in aspects of early development, although not specifically Frizzled-1 or Frizzled-2 signaling. The strategy that was employed (i.e., creating β_2AR/Frizzled chimeras) enabled precise definition of the target genes regulated by activation of a *single* Frizzled, avoiding the pitfalls of the use of conditioned media with variable and indeterminate amounts of active Wnts and other growth factors.

Activation of these Frizzled-specific chimeras also revealed early (e.g., *Lefty1*, *JAB*, *Fce Ig*, *STAM*, and *follistatin*) and

intermediate-to-late (e.g., *MyD118*, *p44 MAPK*) genes that are up-regulated by activation of the Rfz1-pathway specifically and not by activation of the Rfz2-pathway. These target genes are regulated by neither activation of Rfz2 (Table 4) nor treatment of the NCCIT cells with Wnt-3A (Willert et al., 2002). Lefty1 regulates TGFβ signaling (Ulloa and Tabibzadeh, 2001; Tabibzadeh, 2002), whereas STAM and JAB are key factors in Janus kinase signaling (Duhe and Farrar, 1998). The up-regulation of STAM by activation of the Frizzled-1–specific chimera was blocked by expression of DN-Tcf4, implicating the Wnt/β-catenin pathway in the up-regulation of STAM. MyD118 (GADD45b), involved in negative growth control and apoptosis (Vairapandi et al., 2002), is up-regulated late in the response to Frizzled-1 chimera activation and is also sensitive to expression of DN-Tcf4. Nip3, a Bcl-2-binding protein involved in apoptosis (Bruick, 2000), up-regulated by activation of either Rfz1 or Rfz2, is also sensitive to DN-Tcf4. The gene profiling of Frizzled-1 revealed in the current study enabled the identification of a cluster of genes whose structures include promoter elements sensitive to elevation of cellular levels of β-catenin. The ability of expressed DN-Tcf4 to block Frizzled-1 activation of these genes and to block the formation of primitive endoderm provides compelling evidence for the essential role of activation of this cluster of genes in development.

Finally, although the genes for the Janus tyrosine kinase-signal transducer and activator of transcription regulator JAB and the homeobox-containing *Msx-1* genes in development displayed a Tcf-like DNA binding motif and were activated by Frizzled-1–specific chimera, expression of DN-Tcf4 had no effect on their activation, revealing β-catenin–independent signaling of Frizzled-1 at the level of gene expression. Activation of the Frizzled-2 chimera, which was not observed to significantly activate Lef/Tcf-sensitive reporter genes in these mouse F9 cells, led to activation of *Msx-1* gene, but with a much later onset than for activation of Frizzled-1.

The value of gene profiling is that it provides a high throughput strategy with which to analyze the regulation of a large number of genes. In the current work, we attacked the problem of gene profiling of the Wnt-Frizzled pathways, which have been largely resistant to study in the absence of active, purified Wnts. Even as the problem of purified Wnts is solved, the question of how to activate a single, specific Frizzled and not a collection of Frizzleds that may share the ability to be activated by one or more Wnts remains daunting. The GPCR chimera strategy employed for these studies provides an invaluable approach toward dissecting the Frizzled-specific pathways, pharmacologically teased one from the other. We have used Frizzled-specific chimera, DNA microarray, real-time PCR and protein analysis in tandem with the Frizzled-1-specific and Frizzled-2-specific chimeric receptors to gene profile Frizzled signaling. A new appreciation for Frizzled-1-specific versus Frizzled-2-specific regulation of gene expression has emerged using this strategy. These studies demonstrate a proof-of-concept that will enable dissection of the individual roles that Frizzleds play in gene expression, development, and human disease.

Acknowledgments

We thank John Schwedes (University DNA Microarray Facility) and Erich Bremer (Computational Resources, Department of Pharmacology) for their expert and tireless assistance in the DNA microarray and data analysis, respectively.

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