INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

Intracellular signals underlying the inductive effects of agrin during neuromuscular junction formation -

Study on the roles of ras and Shc

Mathieu Lemaire
McGill University, Montreal

Submitted in November 1999

Resubmitted in final form in February 2000

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirement of the degree of Masters' of Sciences.

TABLE OF CONTENTS

Chapter 2 – Materials and Methods

Chapter 3 – Shc interacts with but does not signal downstream of MuSK

Chapter 4 – Ras plays a role during acetylcholine receptor aggregation

Chapter 5 – References

Abstract

Agrin triggers the subsynaptic aggregation of acetylcholine receptor (AChR) via activation of the receptor tyrosine kinase MuSK (muscle-specific kinase). At present, the intracellular mechanisms utilized by MuSK to initiate such a complex process remain unknown. In the present study, I first tested if H-ras was involved in the process of synaptogenesis induced by agrin. The data presented suggest that ras could have a role in this process because a dominant inhibitory ras mutant (ras-N17) partially blocked the inductive effects of agrin while two activated ras mutants (ras-V12 and ras-V12-D38) induced agrin-independent AChR clusters. These effects were not due to major alterations in the levels of AChR, though more experiments are required to confirm these preliminary findings.

Second, I investigated whether the adaptor protein Shc was a downstream effector of activated MuSK. MuSK and Shc could be co-immunoprecipitated, but this association was not consistently observed nor was it modulated by agrin at all times. Generally, no alteration in Shc phosphotyrosine content was observed in response to agrin, and when an increase was detected, it was modest. Finally, agrin did not modulate the interaction between Shc and Grb2. Based on these results, I conclude that Shc interaction with MuSK is not regulated by agrin.

Résumé

L'agrine déclenche l'aggrégation sous-synaptique des récepteurs à l'acétylcholine (RACh) via l'activation du récepteur à tyrosine kinase MuSK. Les mécanismes intracellulaires utilisés par MuSK pour parvenir à former la jonction neuromusculaire sont toujours largement inconnus. Dans cette étude, j'ai tenté de determiner si H-ras est en aval de la cascade signalétique de MuSK. Les données présentées suggèrent que ras pourrait avoir un rôle dans le processus d'agglomération des RACh car un mutant dominant-inhibiteur (ras-N17) bloque la formation de larges aggrégats de RACh induite par l'agrin alors que deux mutants dominant-actifs (ras-V12 et ras-V12-D38) provoquent la formation de ces aggrégat en l'absence d'agrine. Ces effets ne semblent pas dûs à une altération majeure aux niveaux de RACh à la membrane. Étant donné que ces résultats sont préliminaires, plus d'expériences sont requises pour conclure sur cette question.

Deuxièmement, j'ai tenté de déterminer si la protéine adaptatrice Shc interagissait avec MuSK lorsque celui-ci est activé par l'agrine. MuSK et Shc furent co-immunoprécipités en quelques occasions, mais cette interaction n'était pas toujours modulée par l'agrine. Généralement, l'agrine ne provoque pas de changements des niveaux de phosphotyrosine de Shc, et lorsqu'une augmentation est détectée, elle est modeste. Finalement, l'agrine ne provoque pas une augmentation de l'association entre Shc et Grb2. En conséquence, il semble peu probable que l'interaction observée en quelques occasions entre Shc et MuSK ait un rôle important dans le processus de synaptogénèse induit par l'agrine.

Abbreviations

AChE	acetylcholinesterase
AChR	acetylcholine receptor
Akt	serine/threonine kinase akt (also known as PKB)
α-BTX	alpha-bungarotoxin
ARIA	acetylcholine receptor-inducing activity
BDNF	brain-derived neutrophic factor
CAR	Coxsackie-adenovirus receptor
cDNA	complementary DNA
CMV	cytomegalovirus
DG	dystroglycan
DI	dominant inhibitory
DMEM	Dulbecco's modified Eagle's medium
DP	dominant positive
EGF	epithelial growth factor
EGFR	epithelial growth factor receptor
erbB	EGF receptor-like
Erk	extracellular regulated kinase
FGF	fibroblast growth factor
Gab	Grb2-associated binder
GalNAc	N-acetyl-galactosamine
GAP	guanine nucleotide activation factor
GDI	guanine nucleotide dissociation inhibitor
GDP	guanine nucleotide diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GLUT4	glucose transporter
Grb2	growth factor receptor binding protein 2
GTP	guanine nucleotide triphosphate
GTPase	guanine nucleotide triphosphatase
IGF	insulin-like growth factor
IGFR	insulin-like growth factor receptor
IR	insulin receptor
IRS	insulin receptor substrate

MAPK	Mitogen activated protein kinase
MASC	MuSK-accessory specificity component
Mb	myoblast
MEK/MKK/MAPKK	mitogen activated kinase kinase
Mn	motoneuron
mRNA	messenger RNA
Mt	myotube
MuSK	Muscle-specific kinase
Neu	neu differentiation factor
NMJ	neuromuscular junction
NT	neurotrophins
NT-4	neurotrophin 4
PAGE	polyacrylamide gel electrophoresis
PDGFR	platelet-derived growth factor receptor
PKC	protein kinase C
PI3K	phosphoinositol-3 kinase
PLCγ	phospholipase Cγ
p70S6k	ribosomal p70 S6 kinase
PTB	phosphotyrosine binding
RalGDS	ral guanine nucleotide dissociation stimulator
Rapsyn	receptor associated protein at the synapse
ras	transforming protein of Harvey sarcoma virus
RATL	rapsyn-associated transmembrane linker
ROR	Regeneron orphan receptor
RPTP	receptor tyrosine phosphatase
RTK	receptor tyrosine kinase
Shc	src homology 2 and collagen homology protein
SHP	src homology 2 containing protein tyrosine phosphatase
SH2	src-homology 2 domain
SH3	src-homology 3 domain
Sos	son-of-sevenless
TGFb	transforming growth factor b

Chapter 1

General literature Review

General literature review

The neuromuscular junction (NMJ) has been used extensively in the past 25 years as a working model for synapse formation due to its inherent simplicity and experimental accessibility. Significant efforts have been directed toward unravelling the molecular mechanisms and effector molecules involved in postsynaptic differentiation. This led to the discovery of many proteins that are part of the subsynaptic complex (Hall and Sanes, 1993; Burden, 1998; Sanes and Lichtman, 1999). Although our understanding of neuromuscular synaptogenesis has progressed tremendously, several aspects still remain relatively unexplored. In particular, efforts to elucidate the intracellular signaling pathway underlying this process have only provided us with a few scattered fragments of information (Colledge and Froehner, 1998a; Wallace, 1996). In the present study, I have attempted to determine if the signaling proteins ras and Shc have a role downstream of agrin/MuSK signaling during NMJ formation. Below, I discuss these two proteins, followed by an introduction to the concept of primary myogenesis. Then, a detailed description of neuromuscular junction formation will be provided. The signaling processes involved in AChR subsynaptic aggregation and transcription will be emphasized.

Part 1 - Ras

Ras (transforming protein of Harvey sarcoma virus), a small G-protein of 21kD, has been implicated in many signaling pathways activated by extracellular ligands, leading, among other things, to changes in proliferation or differentiation (Boguski and McCormick, 1993). All mammalian cells studied thus far contain three homologous but distinct ras genes: N-ras, Ha-ras and K-ras (Bourne et al., 1990). Analysis of the different ras genes shows that some domains are conserved from yeast to human. Not surprisingly, mutagenesis within these domains significantly alters ras signaling, suggesting that they harbor crucial functional regions. These are the GTPase domain, the GTP binding domain and the switch region 1 and 2 (or effector binding domain; Shih 1986; Polakis 1993). Another vital site is cysteine-286, which is required for ras membrane attachment, a prerequisite for ras activation (Seabra, 1998).

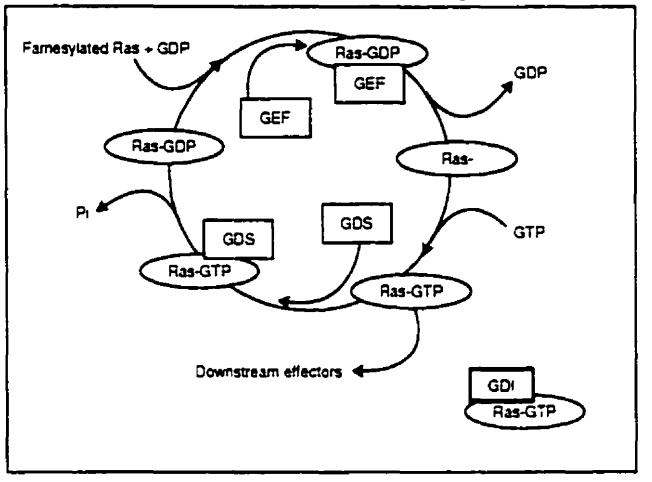
Figure 1.1 A **The Ras GTPase cycle**. Ras are bound most of the time by guanine nucleotides GDP or GTP. ras•GDP, the inactive form of ras, is also the most abundant species. Activation of ras is dependent on the removal of GDP from ras followed by GTP binding. This is catalyzed by ras-GEFs. ras•GTP is only transiently formed since ras possesses an intrinsic GTPase activity that cleaves GTP to GDP and this activity if enhanced by ras-GDS. Ras activity can be abrogated by ras-GDI, which removes ras•GTP from the membrane fraction. Abbreviations used : GEF, guanine nucleotide exchange factor ; GDS, guanine nucleotide dissociation stimulator ; GDI, guanine nucleotide dissociation inhibitor ; GDP, guanine nucleotide diphosphate; GTP guanine nucleotide triphosphate (This figure is modified from Denhardt 1996).

Figure 1.1 B **Schematic of ras activation by a receptor tyrosine kinase**. This representation illustrates the mechanism by which ras is activated by a signaling cascade initiated by the binding of an extracellular ligand to a receptor tyrosine kinase. Tyrosine phosphorylation resulting from dimerization of the RTK creates docking sites for adaptor proteins such as Grb2. Since Grb2 is constitutively complexed with the ras-GEFs called Sos, it is also translocated to the membrane fraction, a perfect location to allow it to modulate ras activity. Quiescent ras, which is bound to GDP, does not interact with downstream effectors. In contrast, the binding of GTP to ras causes major conformational changes that allow signaling molecule to interact with it.

Figure 1.1 C **Typical ras signaling cascade downstream of RTKs**. Activation of RTKs recruits the complex of Grb2/Sos to the membrane directly or via Shc, and promotes ras activation. Ras activity depends on the balance between the activities of positive (ras-GEFs) and negative (ras-GDS) modulators. The primary effectors bind to ras•GTP directly (PI3K, ralGDS, raf-1) and initiate distinct signal transduction cascades. A negative feedback loop has been reported whereby activation of MAPK downstream of ras results in the dissociation of Grb2/Sos complex.

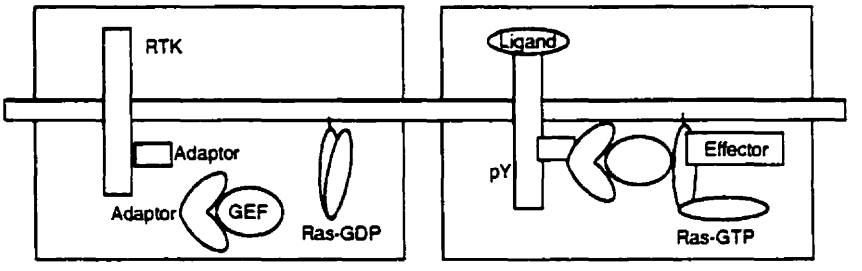
A

Ras GTPase cycle



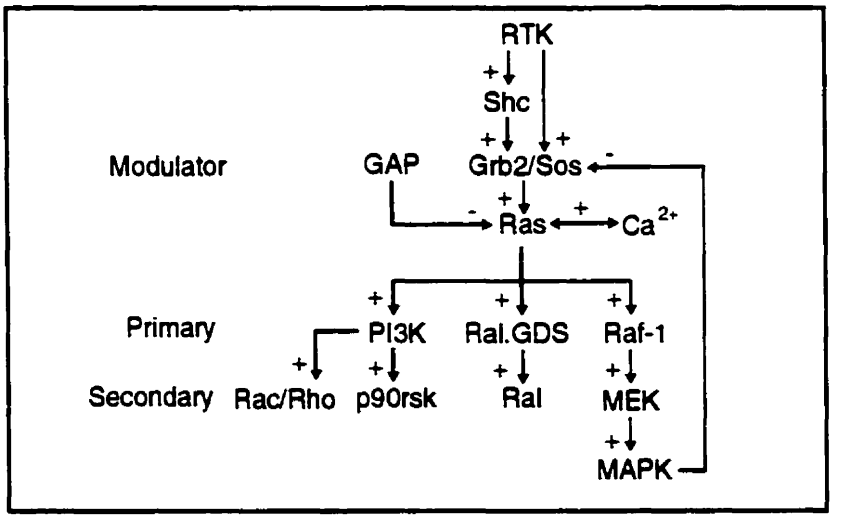
B

Ras activation



C

Ras cascade



At all times, ras proteins are bound by one of two different guanine nucleotides. Interaction between the ras•GTP binding domain and GDP or GTP results in low or high activity levels, respectively (Bourne et al., 1990; Boguski and McCormick, 1993). Indeed, the binding of GTP to ras results in major conformational changes involving mainly switch regions 1 and 2 (Pai et al., 1989; Milburn et al., 1990) which allows downstream signaling molecules such as raf-1 (Daum et al., 1994; Avruch et al., 1994), PI3K (Rodriguez-Viciana et al., 1994) or ralGDS (Kikuchi et al., 1994), to interact with the effector region (figure 1.1 B; for reviews, Marshall 1996; Herrmann 1996). Specific mutations in this domain results in partial loss of ras interaction with downstream effectors (White et al., 1995; Joneson et al., 1996; Khosravi-Far et al., 1996). Ras activity is transient as it is initiated intermittently by tightly regulated signaling pathways and is rapidly terminated due to an intrinsic GTPase activity that hydrolyzes GTP to GDP (Bourne et al., 1991). Thus, ras constantly oscillates between the GDP- and GTP-bound states and acts as a regulatory switch for all signaling cascades that converge onto it (for reviews, Satoh et al., 1992; Rommel and Hafen, 1998).

Three families of proteins directly modulate this cycle by driving it toward or away from the active state (figure 1.1 A). GEFs (guanine-nucleotide exchange factors) such as Sos, promote GDP disengagement and GTP binding (for review, Quilliam et al., 1995). GAPs (guanine-nucleotide activating proteins), like rasGAP, increase ras GTPase activity (for review, Polakis and McCormick, 1992). Finally, GIDs (guanine-nucleotide dissociation inhibitor) cause ras•GTP to dissociate from the membrane and transport it to the cytosol, thus rendering it inactive (for review, Olofsson, 1999). The balance between the activities of these modulatory proteins determines what fraction of ras is active at any given time. Mutations that render ras insensitive to GEFs or GAPs have been identified: ras-N17 (where Ser-17 is changed to Asn-17) is locked in the GDP-bound state and is considered as dominant-inhibitory (Szeberenyi et al., 1990; Cai et al., 1990) and ras-V12, (Gly-12 to Val-12) which cannot hydrolyze GTP, acts as a dominant positive (Beitel et al., 1990; Han and Sternberg, 1990).

It has been known for a long time that ras is involved is signaling downstream of receptor tyrosine kinases (RTKs; figure 1.1 C; for reviews, Burgering et al., 1993;

Pawson, 1994). The most studied signaling pathway implicating RTKs and ras is undoubtedly the ones involving Grb2 and Sos, two proteins that are thought to be constitutively complexed via Grb2 SH3 (src homology 3) domains (Simon et al., 1993). Briefly, autophosphorylation of RTKs triggered by extracellular ligands creates a docking site for Grb2, which binds to it via its SH2 (src homology 2) domain (Lowenstein et al., 1992; Schlessinger, 1993). Grb2 translocation to the cell membrane brings Sos in close contact with ras, thus allowing modulation of ras activity in a RTK-dependent fashion (Buday and Downward, 1993; Li et al., 1993; Chardin et al., 1993; Olivier et al., 1993). Ras signaling is also regulated by a negative feedback loop that leads to Grb2/Sos complex dissociation via MAPK-dependent phosphorylation of Sos on serine or threonine residues (Chemiack et al., 1995; Waters et al., 1995a; Waters et al., 1995b; Rozakis-Adcock et al., 1995).

Over the years, a wide variety of functions have been ascribed to ras and its effectors, ranging from initiation of gene transcription, to cytoskeletal rearrangements, cell survival, proliferation and differentiation (for review, Macara et al., 1996). Not surprisingly, mutations that affect the regulation of this versatile enzyme are often linked to cellular transformation and are found in 30% of all cancers (for review, Barbacid, 1987). Ras seems to be perfectly located within signaling cascades to act as a nodal point for integration of extracellular signals that modulate cellular processes in a co-ordinated manner. Indeed, despite simultaneous convergence of many RTK signaling pathways on one molecule, ras activity still gives rise to a variety of cellular outcomes (for reviews, Vojtek and Der, 1998; Denhardt, 1996). This specificity is likely to be due to the diversity of ras modulators and downstream effectors (for reviews, Wittinghofer and Herrmann, 1995; Parrini et al., 1997).

Part 2 - Shc

The intracellular protein Shc (src-homology and collagen) is ubiquitously expressed, with three different forms (Pelicci et al., 1992). The 47 and 55 kD forms mediate signaling downstream of RTK while the 66kD is thought to act as a negative regulator (Pelicci et al., 1992; Okada et al., 1997). Shc, like Grb2, is said to be an adaptor

protein because it has a modular architecture composed of multiple binding domains allowing it to form multiprotein complexes (for review, Birge et al., 1996). Although it has no intrinsic enzymatic activity, constitutive Shc overexpression induces transformation in NIH 3T3 mouse fibroblasts and results in tumorigenesis in nude mice (Pelicci et al., 1992). Shc possesses two distinct domains that bind phosphorylated tyrosines: a PTB (phosphotyrosine binding) domain (for review, Borg and Margolis, 1998; Kavanaugh and Williams, 1994; Zhou et al., 1995) and an SH2 domain (for review, Ponzetto, 1998; Pelicci et al., 1992), located at the amino- and the carboxy-terminus, respectively (figure 1.2). The SH2 domain binds preferentially to YXXM motifs while the PTB domain interacts with NPXY, where X stands for any residue (Songyang et al., 1993). For the latter, residues surrounding the motif NPXY determine the strength of the interaction while the identity of the X appears to have no effect (He et al., 1995; Laminet et al., 1996). Although the PTB domain is clearly used to interact with activated receptor tyrosine kinases, the role of the SH2 domain remains unclear (Borg and Margolis, 1998). Association of Shc with activated RTK generally results in tyrosine phosphorylation of Shc in the collagen-homology domain (Rozakis-Adcock et al., 1992) and these modified residues constitute new docking sites for signaling molecules that have SH2 domains, such as Grb2 (VanderKuur et al., 1995).

Shc signaling often involves but is not restricted to activation of the ras/raf-1/MAPK pathway via Grb2/Sos (Bonfini et al., 1996). Indeed, association of Shc with activated RTK leads to the translocation of the Grb2/Sos complex to the membrane fraction (figure 1.1 C; Egan 1993). Shc is also able to interact with many other proteins like SHIP (Lamkin et al., 1997), adaptins (Okabayashi et al., 1996) or IRS-1 (Kasus-Jacobi et al., 1997). In most tissues, Shc is simultaneously involved in the signal transduction cascade downstream of many RTK such as the EGF receptor (Batzer et al., 1994), the insulin receptor (Sasaoka et al., 1994) or the IGF-1 receptor (Chow et al., 1998). Notably, Shc-dependent signaling pathways activated by these receptors result in completely distinct cellular processes, illustrating its functional diversity (Cattaneo and Pelicci, 1998).

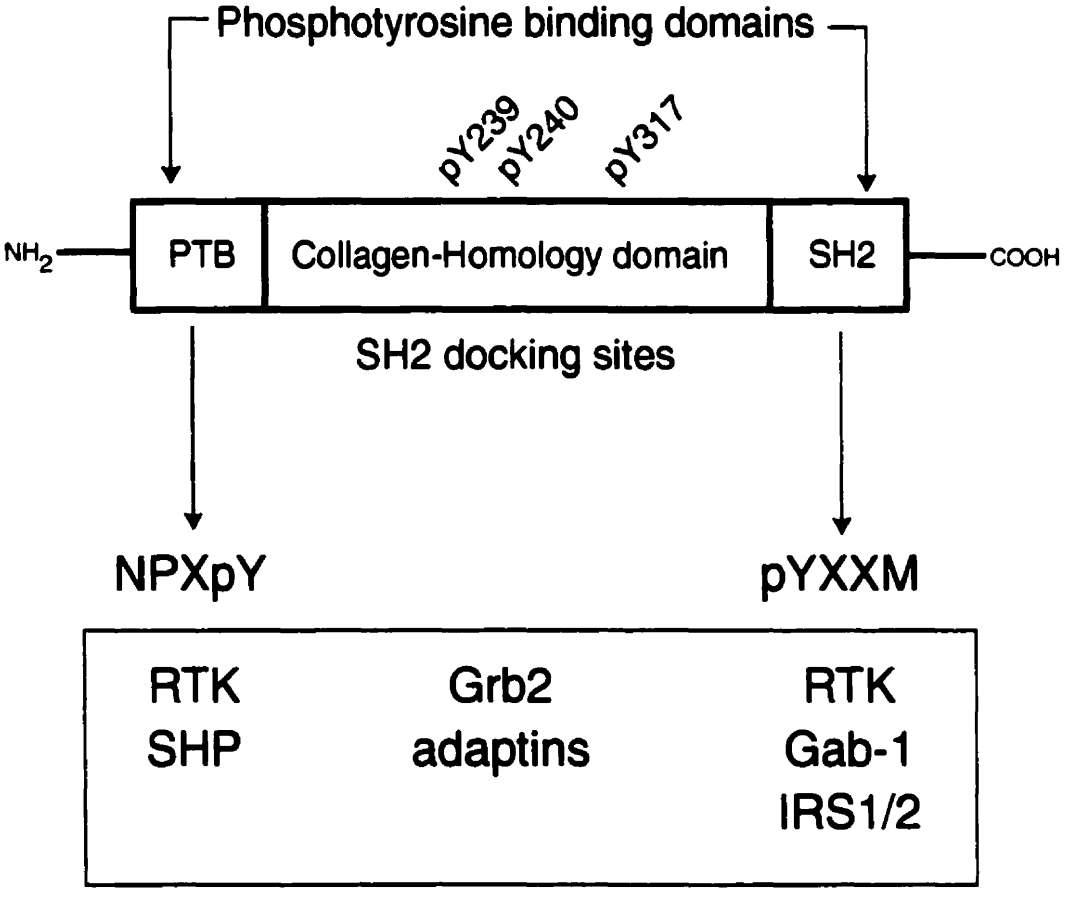

Phosphotyrosine binding domains
pY239
pY240
pY317
NH2
PTB
Collagen-Homology domain
SH2
COOH
SH2 docking sites
NPXpY
pYXXM
RTK
SHP
Grb2
adaptins
RTK
Gab-1
IRS1/2

Figure 1.2 **The domains of Shc**. The structure of Shc is illustrated with the protein tyrosine binding (PTB) domain at the amino terminus and the src homology-2 (SH2) domain at the carboxyl terminus. In the middle, the collagen homology domain is rich in serine-proline residues and contains 3 potential SH2 docking sites at tyrosine 239, 240 and 317. The consensus motifs for the binding of the PTB and SH2 domains are indicated. In the bottom panel are presented examples of proteins that interact with the three functional domains of Shc.

Part 3 - Myogenesis

Skeletal myoblasts (Mb), which originate from the dorsal mesoderm during development, are cells that have the ability to perform two mutually exclusive but complementary tasks. First, they can proliferate, resulting in clonal expansion of the cell population. Second, Mb can exit the cell cycle, start to differentiate and ultimately undergo cell fusion to generate multinucleated myotubes (Mt). This process is referred to as primary myogenesis (for review, Rosenthal, 1989). It is well established that Mb and Mt express very different sets of proteins that can be used as markers to distinguish differentiation levels. For example, Mb possess a wide range of cell cycle-related proteins like the cyclins and cyclin-dependent kinases (cdks) whereas Mt do not (Zhang et al., 1999). In contrast, myosin heavy chain and creatine kinase are only found in Mt (Chamberlain et al., 1985; Lawrence et al., 1989).

Myogenesis regulation

An array of positive and negative cues tightly regulates myogenesis. For example, insulin (Florini et al., 1996) and neuregulin/GGF2 (Kim et al., 1999) promote myogenesis while TGFb (Olson et al., 1986) and FGF (Olson et al., 1987) inhibit it (figure 1.3 A; for review, Olson 1991). Since myocytes are exposed to many such signals during development, signal integration is likely to occur via intracellular signaling cascades. Not surprisingly, many signaling proteins like PI3K (Jiang et al., 1998; Jiang et al., 1999; Calera and Pilch, 1998; Kaliman et al., 1996), MAPK (Bennett and Tonks, 1997), PKC (Vaidya et al., 1991), raf-1 (Dorman and Johnson, 1999), rac1 (Gallo et al., 1999), rho (Takano et al., 1998; Wei et al., 1998; Ramocki et al., 1997) and ralGDS (Ramocki et al., 1998) have all been implicated in myogenesis, and their involvement strongly suggests an important role for ras during myogenesis. Indeed, dominant positive ras is very effective at inhibiting myogenesis (Olson et al., 1987; Payne et al., 1987; Gossett et al., 1988; Sternberg et al., 1989; Konieczny et al., 1989; Vaidya et al., 1991; Weyman et al., 1997), and this effect is due to the secretion of an inhibitory factor that is not TGFb nor FGF (Weyman and Wolfman, 1997). To my knowledge, the effects of expressing dominant inhibitory ras on myogenesis have not been documented (see Results).

A

Myogenesis regulation

Differentiation signals	Proliferative signals
PI3K	Fetal bovine serum
Integrins	bFGF2
Neuregulin (GGF)	TGFbeta1
IGF-1	Ha-Ras
IGF-2	PKC
Insulin	RalGDS

B

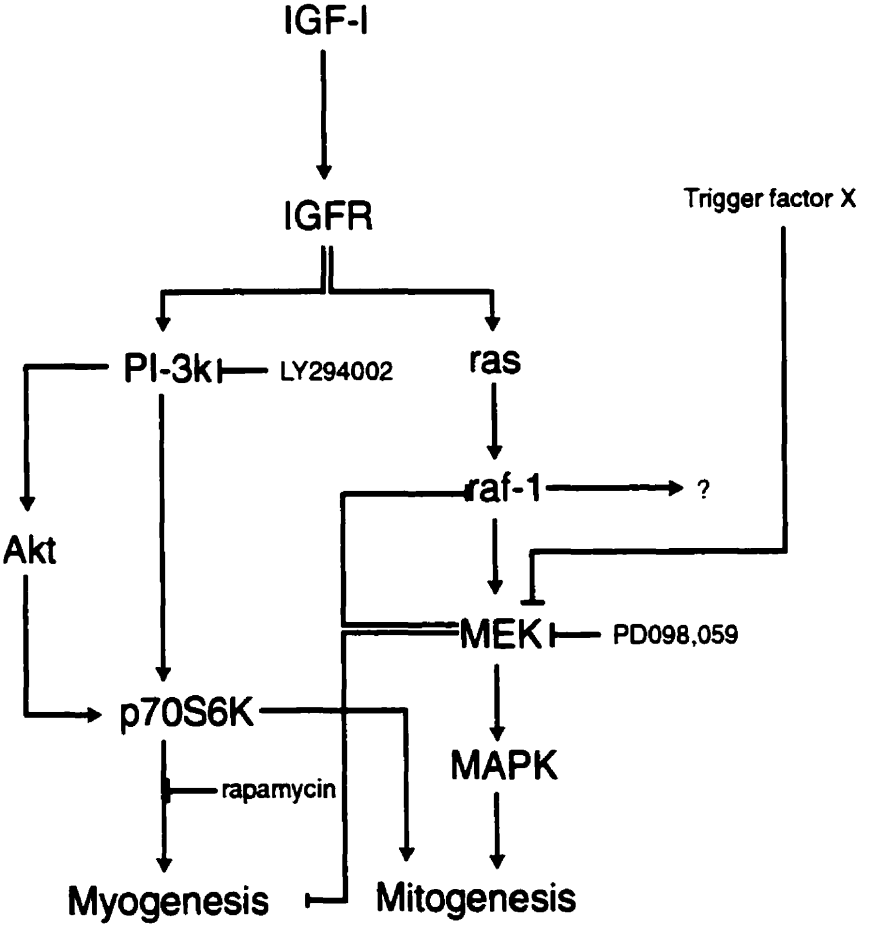


Figure 1.3 A **Factors regulating myogenesis.** Myogenesis is the process by which myoblasts undergo fusion to form multinucleated myotubes. In essence, myoblasts are proliferative cells whereas myotubes are quiescent and fully differentiated entities. Presented here are some of the factors that are known to promote or abrogate myotube formation.

Figure 1.3 B **Regulation of myogenesis by insulin-like growth factor-I.** The effects of insulin-like growth factor-I (IGF-I) on myogenesis are peculiar because depending on the time scale, it either inhibit it (short time) or promotes it (longer exposure). The inhibitory component of the signaling pathway involves mainly the ras/raf-1/MEK/MAPK pathway and results in proliferation (mitogenesis). The positive signals are transduced by PI3K via Akt and p70S6k. For more details, see text and also ref. Coolican et al., 1997.

A role for ras in myogenesis

Recently, the role of ras during myogenesis has been clarified in studies focusing on the modulatory effects of insulin-like growth factor-1 (IGF-1) on myodevelopment (Coolican et al., 1997). This growth factor, which activates IGFRs, displays a peculiar functional dichotomy as it promotes both proliferation (after 1 day of exposure) and differentiation (after 3 days) of myocytes (figure 1.3 B). IGF1-dependent myocyte proliferation has been linked to the ras/raf-1/MEK/MAPK cascade while the IGF1 inductive effects on myogenesis appear to rely on the PI3K/Akt/p70S6k signaling pathway. Exposure of myoblasts to the MEK inhibitor PD098059, in conjunction with IGF1, inhibited IGF1-dependent proliferation but resulted in differentiation levels that were significantly higher than that obtained with IGF1 alone. On the other hand, treatment with either LY294002 (PI3K inhibitor) or rapamycin (p70S6k inhibitor) under similar conditions resulted in an insignificant decrease in cell proliferation but completely prevented IGF1-dependent differentiation. Finally, both LY294002 and rapamycin hindered the myogenic effect of PD098059. From these results, it appears that myogenic factors might modulate the effects of IGF1 on muscle cells by alleviating the negative influences exerted by the ras pathway. Still, blockade of this inhibitory pathway is not sufficient to induce myogenesis since the PI3K/p70S6k pathway is required.

Part 4 - The neuromuscular junction

Motoneurons (Mn) are cells located in the ventral horn of the spinal cord that send very long axons to every skeletal muscle of the body. Once Mn have arrived in the vicinity of the muscle, they send off many projections that innervate the muscle fibers. Although multiple innervation is observed early in development, later on, each myofiber has only one NMJ (for review, Sanes and Lichtman, 1999). The most striking postsynaptic change concerns acetylcholine receptors (AChR) localization and expression. AChR are mobile and uniformly distributed on the myotubes before innervation, however the presence of a Mn in the vicinity induces aggregation of pre-existing AChR subsynaptically (Salpeter et al., 1988). Nerve-muscle contact also results in an overall increase in AChR transcription

that becomes gradually restricted to synaptic nuclei (Klarsfeld et al., 1991; Sanes et al., 1991). It also triggers a switch in AChR subunit stoichiometry, as development proceeds from the embryonic $\alpha 2\beta\delta\gamma$ to the adult $\alpha 2\beta\delta\varepsilon$ (Mishina et al., 1986; Gu and Hall, 1988). A number of elements contained in the basal lamina, on the muscle membrane and in the cytoplasm, are also substantially enriched at the NMJ (figure 1.4). Before discussing the prevailing mechanistic viewpoints underlying AChR localization and expression, the basic structure of a mature NMJ will be described.

4.1 Structural components of NMJ

Basal lamina components

As myotubes form they are gradually ensheathed by an extracellular matrix (ECM) layer known as the basal lamina (BL). It is composed of an array of specific isoforms of molecules like laminin (Patton 1997), entactin (Chiu and Ko, 1994) and collagen (Sanes and Hall, 1979), which form a long-lasting scaffold as it escapes the normal cellular turnover (Slater, 1990). Early on it was observed that factors regulating neuromuscular synapse differentiation were tightly linked to the BL. Indeed, an injured muscle growing back within the remaining BL always formed a normal postsynaptic apparatus at the original synaptic site, even in the absence of reinnervation (Sanes et al., 1978). Likewise, lesioned Mn grew back in their original synaptic location, even when muscle regeneration was prevented. These events were prevented when the basal lamina was removed (Sanes et al., 1978). Some BL residents, in particular certain collagen and laminin isoforms, are dramatically enriched at the NMJ (Sanes, 1982). These proteins are involved in extensive protein-protein interaction thus forming a dense meshwork of homo- and hetero-oligomers in which molecules secreted by Mn and Mt, like ARIA (acetylcholine receptor-inducing activity) or agrin are effectively trapped (for review, Carbonetto and Lindenbaum, 1995).

Membrane-associated proteins

The membrane directly opposed to the nerve terminal is quite different from the rest of the myotube as it is composed subsets of enriched molecules. Typically, one can find transmembrane glycoproteins such as integrins and α/β-DG sarcoglycans aggregated

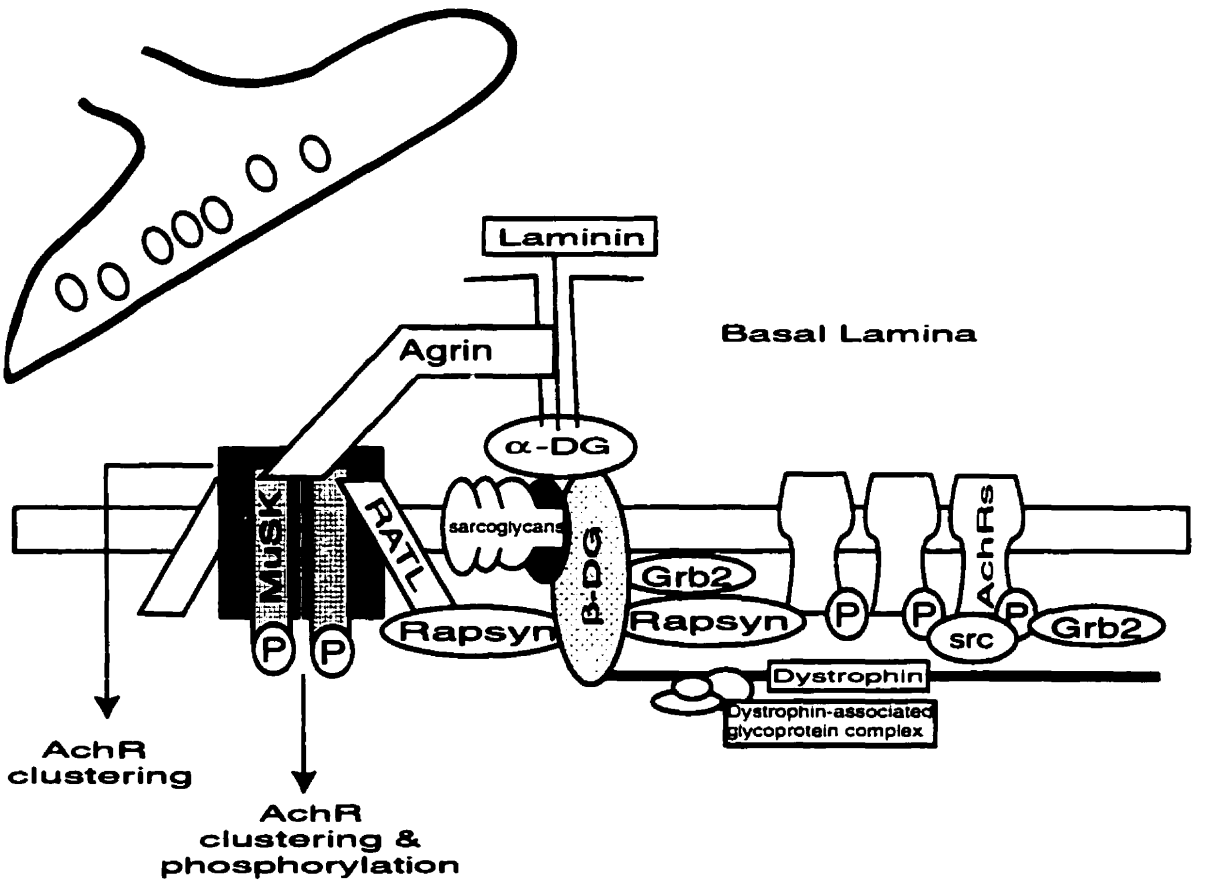

Laminin
Agrin
Basal Lamina
α-DG
MuSK
RATL
sarcoglycans
β-DG
Grb2
Rapsyn
Rapsyn
P
P
P
P
AchRs
src
Grb2
Dystrophin
Dystrophin-associated glycoprotein complex
AchR clustering
AchR clustering & phosphorylation

Figure 1.4 **The proteins aggregated at the neuromuscular junction.** The formation of the subsynaptic apparatus involved the clustering of AChR as well as many other proteins. For simplicity, only the main ones are illustrated. Abbreviations: α/β-DG, α/βdystroglycan; MuSK, muscle-specific kinase; MASC, MuSK-associated signaling component; RATL, rapsyn-associated transmembrane linker; Grb2, growth factor binding protein-2; AchRs, acetylcholine receptors; P, tyrosine phosphorylation, DAG, dystrophin-associated glycoprotein complex.

at the NMJ (reviewed in Carbonetto and Lindenbaum, 1995). Studies with lectins revealed that (unidentified) glycoproteins bearing terminal GalNAcs are also aggregated at the NMJ (Sanes and Cheney, 1982; Scott et al., 1988; Martin and Sanes, 1995). Two types of RTKs are also consistently observed at the NMJ, namely MuSK (Muscle-Specific Kinase) and erbB, the receptors for agrin and ARIA, respectively (Bowen et al., 1998; Zhu et al., 1995). Finally, sodium channels and NCAM are also found at the NMJ, In the more precisely in the throughs of the junctional folds (Flucher and Daniels, 1989; Covault and Sanes, 1986).

Cytosolic proteins

Many intracellular components are accumulated subsynaptically. For instance, the following cytoskeletal proteins are aggregated at the NMJ : rapsyn, utrophin, α-dystrobrevin-1, syntrophin, vinculin, filamin, α-actinin, and actin (Sealock et al., 1984; Peters et al., 1998; Froehner et al., 1997; Shadiack and Nitkin, 1991). A few intracellular messengers, such as PKC-θ (Duca et al., 1998) and cortactin (Peng et al., 1997) are also concentrated subsynaptically. Src-like kinases (Fuhrer and Hall, 1996), which are associated with AChR, might also be recruited at the synapse but it has not yet been shown.

4.2 Modulation of AChR aggregation

The study of the process of AChR clustering was facilitated the use of fluorochrome-labeled α-Bungarotoxin (α-BTX), a non-reversible, high affinity AChR agonist (Barnard et al., 1979). Overnight exposure of cultured myotubes to a molecule is usually sufficient to assess its clustering ability. Early studies revealed that such cluster-promoting agents were present in brain extract but the exact identity remained obscure for a long time (Godfrey et al., 1984). Many purified molecules with aggregating properties have been identified, but their physiological relevance is not always established (see below). First, I will provide a detailed description of the effects of two clustering molecules, namely agrin and laminin, which utilize phosphotyrosine-dependent and -independent pathways, respectively. Finally, it is useful to briefly discuss the other factors

that are known to influence NMJ formation *in vitro*, even though evidences for implication *in vivo* is often lacking.

Agrin

Agrin is a potent postsynaptic differentiation inducer that was isolated and purified from the extracellular matrix of the electric organ of Torpedo (Tsim et al., 1992; Reist et al., 1992). Agrin is a globular protein composed of 9 follistatin-like domains, 4 EGF-like domains and 2 laminin G-domains. Deletion studies have specified the function of some of these domains (Hoch et al., 1994). For instance, the amino terminus binds to laminin (Denzer et al., 1997a), the laminin G-like domains binds to α-dystroglycan (Gesemann et al., 1996) and the C-terminus is required for the clustering activity (Gesemann et al., 1995). The interaction of agrin, a 400kDa heparan sulfate proteoglycan (HSPG), with LN and α-dystroglycan presumably facilitates its stable incorporation into the subsynaptic BL (Denzer et al., 1995). Alternative splicing at three sites (x, y and z) located at the carboxy-terminus generates many agrin isoforms in addition to the unspliced form. Indeed, a 3 or 12 amino acid insert can be added to the x site, a 4 amino acid insert to the y site while inserts of 8, 11 or 19 amino acids can be displayed by agrin at the z site. Since the bioactivity of agrin depends mostly on the inserts y and z, the terminology used corresponds to $agrin_{y,z}$ (Ferns et al., 1992; Ruegg et al., 1992; Ferns et al., 1993; Hoch et al., 1993). I will focus on the two most studied agrin forms, $agrin_{0,0}$ and $agrin_{4,8}$, which have different biological properties.

The first isoform, $agrin_{0,0}$, is secreted by many cell types including neurons and myocytes (Ruegg et al., 1992). At present, the exact function of this isoform (later referred to as muscle agrin) is uncertain, but much evidence suggests that it cannot induce NMJ formation despite the fact that it binds to membrane receptors such as dystroglycan (Sugiyama et al., 1994). Indeed, addition of muscle agrin to cultured myotubes does not promote AChR clustering (Ruegg et al., 1992; Hoch et al., 1993) unless applied at very high concentrations (Ferns et al., 1992), and might in fact reduce AChR tyrosine phosphorylation (Meier et al., 1998a).

In contrast, agrin$_{4,8}$, which is exclusively produced by Mn, is the main orchestrator of NMJ formation (Denzer et al., 1997b; Ruegg and Bixby, 1998). Application of exogenous agrin$_{4,8}$, even in the presence of saturating concentrations of agrin$_{0,0}$, triggers AChR aggregation with other synaptic components (Ferns et al., 1993). Still, these postsynaptic apparati are not fully differentiated, as cultured myotubes exposed to agrin do not form junctional folds, suggesting that agrin may require other signals *in vivo*. Microinjection of agrin$_{4,8}$ cDNA in muscle regions devoid of NMJ lead to the elaboration of an ectopic NMJ, in the complete absence of innervation since neural agrin is secreted by the muscle (Jones et al., 1997; Meier et al., 1997; Cohen et al., 1997). This indicates that the expression of agrin$_{4,8}$ is sufficient to induce NMJ formation.

Mice with a targeted mutation for agrin, which express neither agrin$_{4,8}$ nor agrin$_{0,0}$, have greatly reduced numbers of NMJ (Gautam et al., 1996), yet myotubes derived from these mice are fully responsive to addition of exogenous agrin *in vitro* (Gautam et al., 1999). A recent study elegantly demonstrated the specific role of agrin$_{4,8}$ by generating an agrin$_{4,8}$ isoform-specific knockout mouse (Burgess et al., 1999). Indeed, deletion of the neuronal insert (4,8), resulted in a NMJ phenocopy of the agrin knockout. This proved that agrin$_{4,8}$ is necessary to induce synapse formation while agrin$_{0,0}$ is dispensable (these mice expressed normal levels of agrin$_{0,0}$).

Tyrosine phosphorylation

Further support for the involvement of agrin in NMJ formation stems from its intimate link to tyrosine kinase activity. Indeed, agrin induces sequential tyrosine phosphorylation of MuSK, its putative receptor (see below), and of AChR β-subunit in a dose-dependent manner (Glass et al., 1996a; Ferns et al., 1996). Tyrosine phosphorylation of AChR β-subunit is thought to be important in clustering since tyrosine kinase inhibitors that block MuSK or AChR phosphorylation also compromise their aggregation (Ferns et al., 1996; Wallace, 1994). Tyrosine phosphorylation of the AChR also correlates with the degree of cytoskeletal attachment (Meier et al., 1995), implying that such a modification could be important in stabilizing clusters. Immunocytochemical studies with phosphotyrosine antibodies revealed that phosphotyrosylated proteins are highly

concentrated at the NMJ (Qu et al., 1990; Wallace et al., 1991; Baker and Peng, 1993; Qu and Huganir, 1994). A role for tyrosine phosphatase activity during AChR cluster formation and dispersal has also been suggested from studies with phosphatase inhibitor such as pervanadate (Wallace, 1995; Peng et al., 1995). Despite strong correlative evidence, it appears that tyrosine phosphorylation of AChR-β is not as important as anticipated since replacement of all tyrosine by phenylalanine on the β-subunit does not hinder AChR clustering (Meyer and Wallace, 1998). It is important to understand that this recent finding only challenges the involvement of tyrosine phosphorylation in the final step (AChR clustering) within the current model of MuSK-dependent signaling.

Agrin receptor : MuSK

Even if the importance of AChR tyrosine phosphorylation has been over-estimated, evidence for the involvement of a tyrosine kinase-based signaling cascade during NMJ formation is strong, yet still incomplete. Indeed, a component of the agrin receptor was recently cloned by screening a cDNA library from injured skeletal muscle and is referred to as MuSK (muscle-specific kinase; Valenzuela et al., 1995). Importantly, MuSK is expressed at the right time to be the agrin receptor since it is only observed on developing myotubes (Valenzuela et al., 1995). Furthermore, its expression is restricted to the NMJs in adult myofibers, except after injury or denervation upon which massive transcription occurs resulting in uniform MuSK distribution on the membrane (Valenzuela et al., 1995).

Much evidence suggests that MuSK transduces agrin's signal (for review, Wells and Fallon, 1996). First, $agrin_{4,8}$ specifically induces MuSK activation and co-localization at AChR clusters (Glass et al., 1996b; Hopf and Hoch, 1998). Second, the phenotypes of $agrin_{4,8}$ and MuSK knockout mice are overlapping (they both exhibit a drastic reduction in the number, size and density of NMJs; Gautam et al., 1996; DeChiara et al., 1996) and myotubes cultured from MuSK-/- mice are not responsive to addition of exogenous $agrin_{4,8}$ (Gautam et al., 1999). Third, expression of constitutively active MuSK in extrasynaptic muscle regions results in the formation of functional ectopic postsynaptic differentiation (Jones et al., 1999) whereas expression of a kinase dead MuSK abrogates agrin's effect (Glass et al., 1997; Zhou et al., 1999). Interestingly, when expressed in

heterologous cells, MuSK does not bind to and is not activated by agrin (Glass et al., 1996c). Since MuSK affinity for agrin is very low and occurs only in the context of muscle, the existence of an as yet unidentified MuSK co-receptor, termed MASC (MuSK-Accessory Specificity Component) was proposed (Glass et al., 1996a).

Structurally, the cytoplasmic tail of MuSK is highly homologous to the equivalent domain of Trk receptors, suggesting that MuSK might signal through a Trk-related signal transduction cascade (Valenzuela et al., 1995). In particular, they both share a NPXY motif in their juxtamembrane domain, which a docking site for Shc and PLC-γ in the context of Trk (Rozakis-Adcock et al., 1992;Vetter et al., 1991). Mutagenesis of this motif demonstrated that it was crucial for Trk signaling (Stephens et al., 1994; Yoon et al., 1997) and also for MuSK (Zhou et al., 1999). The MuSK ectodomain is very similar to that of Torpedo RTK (Jennings and Burden, 1993) and ROR1/2 (Regeneron Orphan Receptor; (Masiakowski and Carroll, 1992). Unexpectedly, NT-3 (TrkC ligand) activation of a chimeric receptor containing MuSK's intracellular domain and TrkC extracellular portion resulted in AChR tyrosine phosphorylation, but it was unable to promote AChR clustering (Glass et al., 1997). Hence, both MuSK's intra- and extracellular portions are required in order to get proper AChR aggregation, but neither is sufficient on its own. Therefore, MuSK-induced AChR clustering is more complex than originally thought since it appears to involve both the kinase activity and the ectodomain. In order to understand this peculiar behavior, it might be instructive to draw comparison between MuSK signaling and that of FGFR (fibroblast growth factor receptor), since it also uses both domains to promote functional changes in myocytes (Kudla et al., 1998). From these results and the work of Meyer and Wallace (1998) emerges a scheme in which AChR tyrosine phosphorylation is neither sufficient nor required for AChR clustering induced by agrin/MuSK.

Rapsyn

Rapsyn is a cytoskeletal protein that binds to and is co-distributed with AChR (Froehner et al., 1981; Sealock et al., 1984; Bloch and Froehner, 1987; Frail et al., 1988). Since it does not affect the kinetics of the response of AChR to acetylcholine, it was

postulated to play a role in the formation of NMJ (Neubig et al., 1979). A better understanding of its function was gained by expressing rapsyn and putative molecular partners in a heterologous cell system (QT-6 cells, fibroblasts). It is noteworthy that rapsyn molecules are clustered together when expressed alone in these cells (Qu et al., 1996). This approach is validated because of the fact that the candidate proteins are uniformly distributed unless rapsyn is co-expressed. Efficient co-aggregation was observed when rapsyn was expressed together with AChRs, dystroglycan or MuSK but not glutamate receptors (Brennan et al., 1992; Apel et al., 1995; Gillespie et al., 1996). When the pairs AChR/DG or AChR/MuSK were expressed along with rapsyn, the clusters were perfectly coincident, indicating that large complexes had formed (Apel et al., 1995; Gillespie et al., 1996). Interestingly, the interaction between rapsyn and MuSK was sufficient to induce MuSK activity in this system, indicating that rapsyn might play a role in modulation of MuSK signaling (Gillespie et al., 1996): this finding is still controversial as it has not been reproduced by other investigators yet. While the rapsyn binding site on dystroglycan has been mapped to its cytoplasmic tail (Cartaud et al., 1998), it appears that rapsyn interacts with the MuSK ectodomain via an unidentified molecule called RATL (Rapsyn-Associated Transmembrane Linker; Apel et al., 1997). This might explain, at least partially, why the MuSK ectodomain is so important (Glass et al., 1997). Since all these proteins are similarly co-expressed in skeletal muscle but clustering is strictly dependent on agrin, it suggests that agrin/MuSK signaling may relieve intrinsic inhibitory signals in AChR clustering (Sanes et al., 1998).

Rapsyn is required for NMJ formation since rapsyn -/- mice display altered NMJs, however the phenotype is somewhat milder than that of agrin and MuSK knockouts (Gautam et al., 1995). Interestingly, cultured myotubes from these mice form normal subsynaptic aggregates of MuSK, AChE and laminin β2 in response to agrin, suggesting that these are part of a MuSK "primary scaffold" (Apel et al., 1997). Furthermore, agrin induces MuSK but not AChR tyrosine phosphorylation in cultured myotubes from these mice, thus implicating rapsyn in the tyrosine kinase signaling pathway downstream of MuSK (Apel et al., 1997). Thus, both the ecto- and endodomain-dependent signals

originating from MuSK appear to depend on rapsyn as a signaling effector (for review, Glass and Yancopoulos, 1997).

Agrin also binds to dystroglycan and integrins

In vivo, the long-term subsynaptic incorporation and stabilization of agrin in the synaptic cleft cannot be sufficiently accounted for by the indirect agrin-MuSK interaction, suggesting that it requires other factors. One such high affinity factor could be MASC, the putative MuSK co-receptor since its role would be to effectively present agrin molecules to MuSK. While agrin interacts with other ECM components like laminin, it has recently been found to interact with membrane-associated glycoproteins that are co-localized at the NMJ, namely DG (Gee et al., 1994; Campanelli et al., 1994; Sugiyama et al., 1994) and integrins (Martin and Sanes, 1997; Burkin et al., 1998). This is not surprising given that laminin binds to both DG and integrins and has some homology with agrin.

Although DG does not possess an enzymatic domain, DG could potentially be involved in cell signaling since it interacts with the cytoplasmic proteins rapsyn and Grb2 (Apel et al., 1995; Yang et al., 1995). Still, DG does not display a preference for neural agrin (Sugiyama et al., 1994) and agrin fragments that do not bind DG still induce MuSK/AChR phosphorylation and aggregation (Meier et al., 1996). Therefore, DG binding to agrin might be used to stabilize secreted agrin on muscle membrane (for review, Fallon and Hall, 1994). In addition, integrin $\alpha v\beta 1$ binds to agrin but also appears to be a functional agrin co-receptor since blockade using antibodies or antisense oligonucleotides compromised agrin's effects (Martin and Sanes, 1997).

In the following section, a brief review of the other factors known to induce or modulate AChR clustering *in vitro* or *in vivo* is presented. While some of these findings might reveal to be experimental artifacts, it is probable that some of them will soon integrate the model of AChR clustering. I thought it is instructive because some of these findings are often forgotten in the current literature, owing to the lack of strong evidence linking them to the current model of AChR clustering.

Laminin and AChR clustering

Another factor known to induce AChR clustering is laminin. Addition of exogenous laminin to cultured myotubes also induces AChR clustering but this process does not require tyrosine kinase activity (Montanaro et al., 1998). At this point, there is evidence of the involvement of both DG and integrin $\alpha 7\beta 1$ in the process. Indeed, interactions between laminin-1 and DG potentiate the clustering activity of agrin (Montanaro et al., 1998; Sugiyama et al., 1997). In contrast to those results, another group found that blockade of integrin $\alpha 7\beta 1$ inhibited both laminin- and agrin-induced clustering and that integrin $\alpha 7\beta 1$/AChR association was solely dependent on laminin-$\beta 2$ addition (Burkin et al., 1998). Integrin $\alpha 7\beta 1$ knockout mouse have no apparent NMJ defects probably due to compensation by other integrin isoforms. Laminin $\beta 2$ knockout mice have a weak NMJ defect (Patton et al., 1997). Based on these findings, it has been proposed that clustering pathways activated by agrin and laminin are distinct and parallel, co-operating in a synergistic manner. It is impossible at this point to determine if laminin signaling is dependent on DG and/or integrins.

Calcium

It appears that both extracellular and intracellular calcium ions are important for AChR clustering. Indeed, calcium influx induced by muscle activity promotes metabolic stabilization of AChR at the endplate (Rotzler et al., 1991), high extracellular calcium induces AChR aggregation (Bloch, 1983; Mook-Jung and Gordon, 1995) while depletion of calcium destabilizes the clusters (Wallace, 1988; Caroni et al., 1993). It is noteworthy that a rise in extracellular calcium can also induce agrin-independent MuSK activation (Grow et al., 1999). Furthermore, extracellular calcium is required for the binding of agrin to muscle cell surface (Nastuk et al., 1991; Ma et al., 1993). Since the calcium chelator BAPTA blocked the inductive effects of agrin, it has been proposed that agrin triggers intracellular calcium fluxes that are required for AChR clustering (Megeath and Fallon, 1998). Interestingly, BAPTA does not affect agrin-induced AChR β-subunit tyrosine phosphorylation, indicating that calcium-dependent signaling acts either downstream of or in parallel to AChR phosphorylation (Megeath and Fallon, 1998).

Carbohydrates

The importance of carbohydrates on membrane proteins in AChR clustering is only starting to be unveiled despite the fact in 1982, it was reported that membrane glycolipids and glycoproteins harboring terminal GalNAc were located exclusively at the NMJ (Sanes 1982; see also Martin et al., 1999). It was shown that the lectin VVA-B_4, which recognizes GalNAc, could induce agrin-independent AChR aggregates, even in MuSK -/- myotubes (Martin and Sanes, 1995). Furthermore, application of GalNAc conjugated to BSA specifically blocked agrin-induced clusters. Undoubtedly, GalNac is very important for NMJ formation but its function is unclear.

Sialic acid, which is selectively cleaved by neuraminidase is another sugar moiety that might play a role during NMJ formation since exposure of myotubes to neuraminidase induces AChR clustering in the absence of agrin (Martin and Sanes, 1995; Grow et al., 1999). Similar treatment also induces agrin-independent MuSK activation as well as AChR β-subunit tyrosine phosphorylation (Grow et al., 1999). Since neuraminidase treatment provoked a change in the apparent molecular weight of MuSK, it assumed that removal of terminal sialic acid from MuSK disinhibits its catalytic activity (Grow et al., 1999). A similar mechanism has been reported for TrkA, where TrkA deglycosylation by tunicamycin resulted in ligand-independent activation (Watson et al., 1999). Under such conditions, Shc associated with TrkA but ras/MAPK pathway was not activated, probably because unglycosylated TrkA is retained intracellularly (Watson et al., 1999).

Growth factors

Incubation of myotubes with various growth factors results in AChR clustering but the underlying mechanism remains unknown. For example, both IGF-1 (insulin-like growth factor-1) and bFGF (basic fibroblast growth factor) induce AChR aggregation via a tyrosine kinase activity-dependent mechanism (Peng et al., 1991; Dai and Peng, 1992; Baker et al., 1992; Baker and Peng, 1995). In another study, co-application of bFGF, EGF (epithelial growth factor) and insulin resulted in an increase in AChR clustering (4.5 fold) and transcription (4 fold). When applied individually, only bFGF increased AChR

clusters (3 fold) and insulin raised transcription levels (2 fold; Askanas et al., 1985). A physiologic role for bFGF, EGF, IGF-1 or insulin is uncertain at this point.

HB-GAM (heparin-binding growth-factor-associated molecule) also promotes AChR clustering but has the particularity of being accumulated at hot spots and at NMJs (Peng et al., 1995). HB-GAM also binds to agrin and potentiates its clustering effect (Daggett et al., 1996). This finding is supported by the fact that addition of heparin, heparan sulfate or suramin (a heparin analogue), which is thought to inhibit agrin binding, prevents AChR clustering (Wallace, 1990; Hopf and Hoch, 1997). In the nervous system, the interactions of HB-GAM are extremely complex as it binds many heparan sulfate proteoglycans, for example, N-syndecan (Lauri et al., 1999), phosphacan/PTPζ (Milev et al., 1998) and RPTPζ/β (Maeda et al., 1999). RPTPζ/β is a receptor tyrosine phosphatase that interacts with PSD95 in neuronal postsynaptic densities (Kawachi et al., 1999). Nevertheless, based on the *in vitro* findings, HB-GAM may be a physiologically relevant clustering modulator.

Src-like kinases

Since tyrosine phosphorylation of AChR appeared to be important for its function and aggegration, it is important to determine which kinases are involved in the process. Src-like kinases are good candidates since they were abundant in the electric organ of rays, an organ that is highly enriched in AChR (Fukami et al., 1986; Swope and Huganir, 1993). Recent work has shown that src-like kinases such as src, fyn or fyk are associated with phosphorylated AChR via SH2 domains, and that AChR are a substrate for these kinases (Swope and Huganir, 1994; Fuhrer and Hall, 1996). Still, neither the AChR/src interaction nor src activity were modulated by agrin (Fuhrer and Hall, 1996). Recently, src was shown to interact also with raspy (Mohamed and Swope, 1999). This association induced src activity and resulted in AChR tyrosine phosphorylation and cytoskeletal anchoring (Mohamed and Swope, 1999). In spite of all this correlative evidence, the function of src kinases during NMJ formation remains unclear. Of note, it was demonstrated that skeletal muscles expressing an activated src (v-src) had impaired AChR clustering (Anthony et al.,

1984), suggesting that src activity, at least when deregulated, is incompatible with normal cluster formation.

Cytoskeletal remodeling: cortactin and rac

One major src substrate, cortactin (Wu et al., 1991), is localized in regions of active actin assembly and is involved in cytoskeletal rearrangements (Wu and Parsons, 1993). Src strongly downregulates the activity of cortactin (Huang et al., 1997). Interestingly, cortactin is also specifically associated with early onset AChR clusters (Peng et al., 1997) and is highly expressed in embryonic skeletal muscle (Wu and Montone, 1998). Hence, it is possible that cortactin activity might promote NMJ formation by altering the subcellular distribution of many cytoskeletal proteins. It is intriguing that the binding of HB-GAM to N-syndecan modulates cortactin-src and cortactin-fyn interactions during neurite outgrowth (Kinnunen et al., 1998).

In light of those findings, it is not surprising that the small GTPase rac is important for NMJ formation as it is known for its involvement in cytoskeletal remodeling downstream of growth factors (Ridley and Hall, 1992). Indeed, agrin-induced clustering was perturbed by expression of dominant inhibitory rac while dominant active rac bypassed agrin's requirement (Weston, C. 1998 Neurosci. Soc. Abstr.). In the same study, both laminin and agrin induced phosphorylation of JNK, a marker for rac activation. Hence rac clearly acts downstream of agrin and laminin and might constitute a point of convergence between these two pathways. It is notable that activation of rac is regulated by calcium fluxes and by src activity and it promotes cortactin translocation from the cytosol to the cytoskeleton (Weed et al., 1998).

A role for autocrine neurotrophins?

Compelling evidence support the notion that the neurotrophins BDNF and NT-4, which are secreted by muscles, negatively regulate agrin-induced AChR clustering via TrkB receptors (Wells et al., 1999). Indeed, inhibition of AChR clustering was observed upon addition of exogenous BDNF or NT-4 (but not NGF or NT-3) or after treatment with an anti-TrkB antiserum, which promotes ligand-independent receptor dimerization.

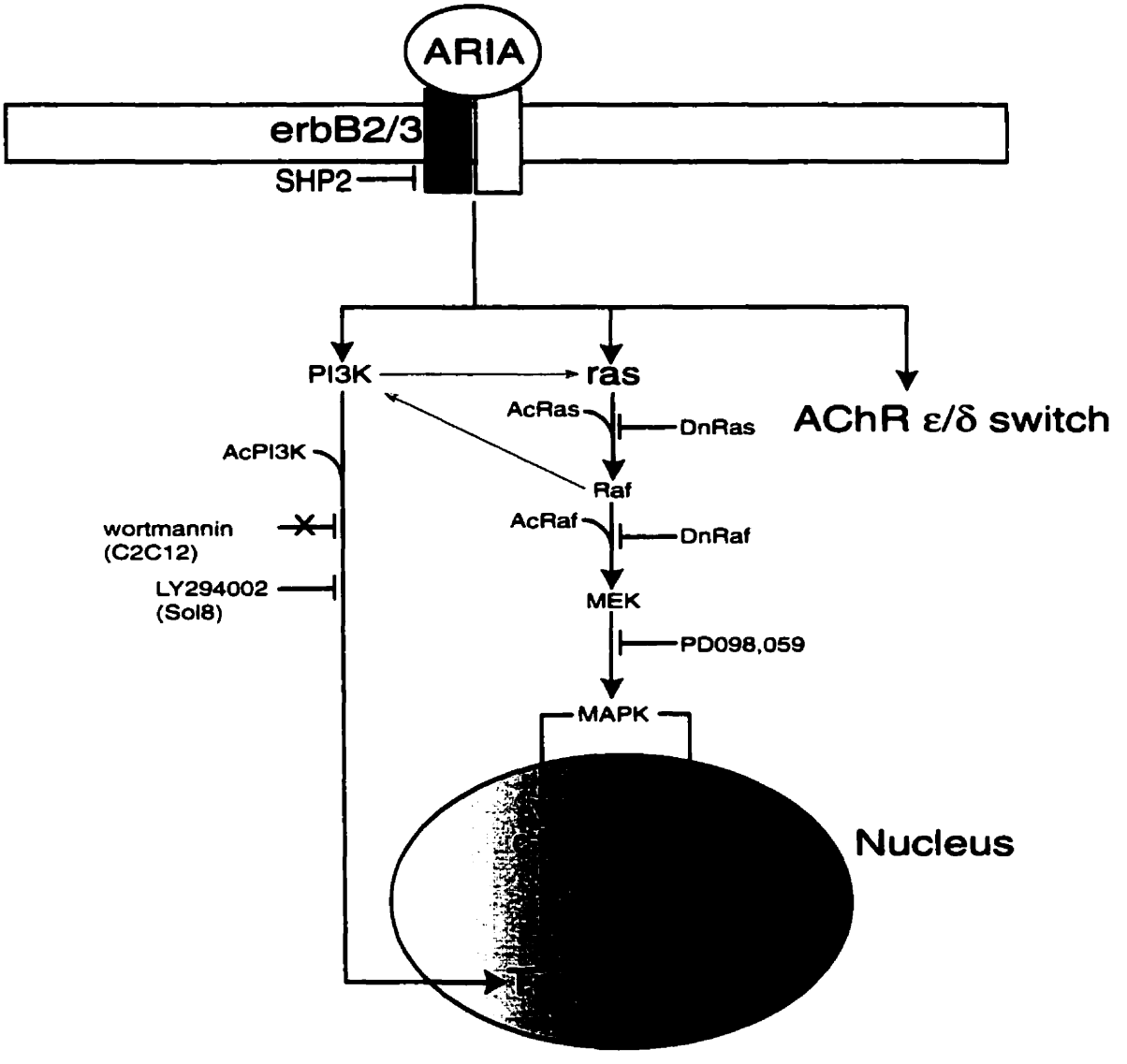
Furthermore, when myotubes were incubated with soluble TrkB receptors that scavenge endogenous BDNF/NT-4, agrin-independent aggregates were visible. Given that TrkB and MuSK have highly homologous cytoplasmic tails, it is tempting to speculate that they are competing for a limited pool of common second messengers, such as PLC-γ, Shc, Grb2/Sos, SNT, ras, MAPK, akt/PKB or PI3K (for review, Kaplan, 1998).

4.3 Modulation of AChR expression

The precise timing of expression of AChR subunits and the strict subsynaptic localization of the AChR mRNA and proteins strongly suggest that all aspects of AChR biology are tightly regulated. Indeed, AChR expression at the membrane can be controlled at the level of mRNA transcription, translation, post-translational modifications, subunit assembly and membrane insertion (for review, Steinbach, 1981). Many neuron-derived factors have been shown to modulate AChR mRNA levels, including CGRP (calcitonin gene-related peptide; (New and Mudge, 1986; Fontaine 1987), ARIA and agrin. The evidence supporting a role for CGRP during NMJ maturation will not be discussed since the CGRP receptor is not aggregated at the NMJ (Jennings and Mudge, 1989) and CGRP null mice have normal AChR transcription at the NMJ (Lu et al., 1999).

ARIA

The best candidate for neuron-derived regulation of AChR expression is the polypeptide ARIA (acetylcholine receptor-inducing activity; Falls et al., 1993), which is part of the neuregulin (NRG) family of growth factors (Lemke, 1996). Although it initiates transcription of all AChR subunits (Si and Mei, 1999), the induction of the epsilon-subunit is by far strongest (Chu et al., 1995), suggesting that ARIA might be responsible for the switch to adult type of AChR (Missias et al., 1996; discussed above). ARIA also increases utrophin and sodium channel mRNA levels (Gramolini et al., 1999; Corfas and Fischbach, 1993) but does not alter that of MuSK, rapsyn, erbB2, erbB3 or SHP-2 (Si and Mei, 1999). Neuregulin-deficient mice are not informative with respect to ARIA's role at the NMJ since they die before synaptogenesis (Meyer and Birchmeier, 1995; Martin et al., 1996). On the other hand, heterozygous mice have a 50% reduction in



Elevated mRNA

AChR (all subunits)
Utrophin

Unchanged

MuSK
Rapsyn
Dystroglycan
erbB2/3
GADPH

Figure 1.5 **Modulation of AChR transcription by ARIA**. ARIA is a factor secreted by neurons and muscle that influences the transcription of AChR when applied to myotubes. ARIA activates a signaling cascade via erbB2/3 RTK that involves both ras and PI3K. The two paths are distinct but not totally independent since cross talk appears to occur (small arrows). SHP2 is a protein tyrosine phosphatase that binds to activated erbB RTK and downregulate its activity. The mutant proteins expressed and the drugs used to delineate this pathway are indicated. At the bottom are the proteins for which mRNA levels were investigated in response to ARIA treatment. Abbreviations : Ac, activated; Dn, dominant-negative. For more details, see text.

al., 1998). Second, the dominant inhibitory ras mutant N17 prevents ARIA-dependent transcription of the epsilon-subunit whereas dominant positive ras (Cook et al., 1993) bypasses the ARIA requirement (Tansey et al., 1996). Third, similar results were obtained using dominant inhibitory and active raf-1 mutants (Tansey et al., 1996). Fourth, MEK/MAPK activity is modulated by ARIA and inhibition of its function by PD098059 prevents the ARIA-dependent increase in AChR transcripts in both Sol8 and C2C12 cells (Tansey et al., 1996; Altiok et al., 1997; Si et al., 1996; Si and Mei, 1999). Inhibition of MAPK by oligonucleotide EAS2 has similar consequences in chick myotubes (Altiok et al., 1997).

ARIA signaling via PI3K pathway

The evidence supporting a role for PI3K pathway downstream of ARIA/erbB in Sol8 cells is the following: 1) PI3K activity is stimulated by ARIA, 2) a dominant positive PI3K bypassed the ARIA requirement and 3) PI3K inhibitor LY294002 prevented the ARIA-induced increase in AChR mRNA (Tansey et al., 1996). Intricate cross talk between the ras and PI3K pathways was reported (Tansey et al., 1996). However, results from two other groups contradict these results. In the first report, the PI3K inhibitor wortmannin failed to inhibit ARIA's effect on C2C12 cells (Si et al., 1996) while in the second, it promoted AChR transcription in chick primary myotubes (Altiok et al., 1997). These conflicting results may reflect differences in cell and/or drugs used and highlight the difficulty in generalizing between systems.

ARIA and tyrosine phosphatases

An ARIA-dependent signaling pathway involving the protein tyrosine phosphatase SHP2 (src-homology 2 domain-containing phosphatase) was recently reported to negatively regulate erbB activity. In C2C12 cells, expression of a dominant positive SHP2 blocked ARIA-dependent AChR transcription while a dominant inhibitory SHP2 enhanced it (Tanowitz et al., 1999). SHP2 only associates with activated erbB receptors (He et al., 1995) and appears to downregulate them via its phosphatase activity. Given its accumulation at the NMJ, SHP2 might be the tyrosine phosphatase activity that is involved

in AChR cluster formation and dispersal discussed above, in addition to its role in the regulation of erbB signaling.

Agrin and AChR transcription

While the role of agrin in AChR clustering has been established for a long time, its implication in transcriptional regulation has only recently emerged. Initial results suggested that agrin could, independently of any neural ARIA inputs, promote the formation of a fully functional NMJ (Jones et al., 1997; Meier et al., 1997; Cohen et al., 1997). Indeed, muscle and neural agrin are equally efficient at inducing AChR β-subunit transcription (Jones et al., 1996), a process which requires immobilization of agrin (Jones et al., 1997). Furthermore, both agrin-/- and MuSK-/- mice have reduced AChR gene expression despite the fact that erbB receptors were not aggregated at the NMJ (Gautam et al., 1995; Gautam et al., 1999). Induction of ectopic synapses by forced-expression of $agrin_{4,8}$ in muscle fibres results in normal NMJ formation in the complete absence of innervation and neural ARIA (Meier et al., 1997; Meier et al., 1998b). Similarly, ectopic expression of a constitutively active MuSK results in agrin-independent NMJ formation with normal AChR transcription (Jones et al., 1999). Recent data indicate that the effects of agrin on AChR transcription might arise from modulation of ARIA/erbB signaling since agrin induces clustering of muscle ARIA and erbB receptors *in vitro* (Rimer et al., 1998). Similar results were obtained by *in vivo* studies, showing that ARIA and erbB receptors were present at ectopic synapses resulting from expression of $agrin_{4,8}$ or activated MuSK (Meier et al., 1998b; Jones et al., 1999). Therefore, it is still unclear whether agrin can itself modulate AChR transcription independently of muscle or neural ARIA.

In conclusion, it appears that a numbers of factors are influencing the formation of the neuromuscular junction induced by neural-derived agrin (figure 1.6). Furthermore, the process of aggregation and subsynaptic transcription are probably very tightly interconnected. Undoubtedly, as our understanding of neuromuscular junction formation progresses, it will prove that this «simple» system is, in fact, extremely complex.

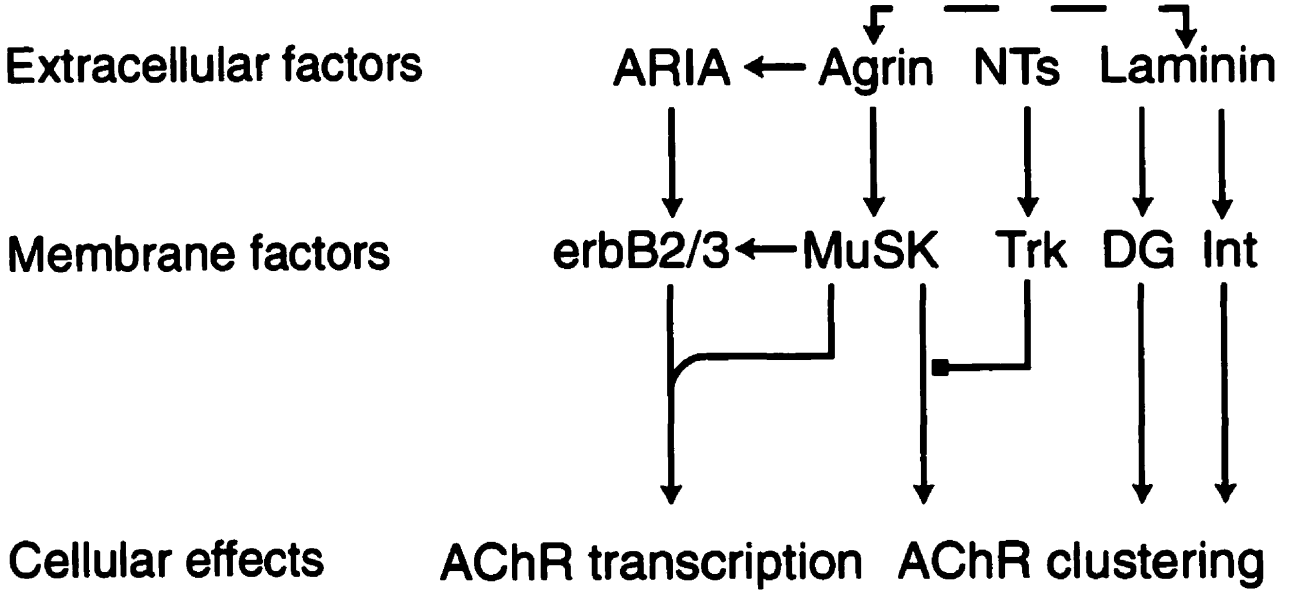

Extracellular factors
ARIA
Agrin
NTs
Laminin
Membrane factors
erbB2/3
MuSK
Trk
DG
Int
Cellular effects
AChR transcription
AChR clustering

Figure 1.6 **Model for the induction of AChR clustering and transcription.** Signaling initiated by the release of agrin from motoneurons modulates AChR clustering. This process requires MuSK and integrins. An alternative pathway is triggered by laminin via integrins and dystroglycan. Neurotrophins (NTs) downregulate agrin-induced clustering by activated TrkB. ARIA is necessary but not sufficient to account for induction of AChR mRNA transcription. Agrin's role might not be restricted to the clustering process as it appears to play an important role in the transcription initiation. Interplay between ARIA and agrin might occur extracellularly since they bind to each other, or in the cytosol via convergence of signaling cascades (erbBs are required for the agrin-dependent induction of AChR transcription). Agrin is also known to interact with laminins, α-dystroglycan and integrins.

Objectives

I attempted to unravel parts of the signaling cascade downstream of the receptor tyrosine kinase MuSK. This study has two main parts. First, I was concerned with determining if the small GTPase ras could play a role in agrin-induced AChR clustering that would be distinct from that activated by ARIA via erbB receptors. My main goal was to try to answer to the following questions:

1) What are the consequences of expressing ras mutants on the levels of AChR on the membrane of myotubes?

2) Does the expression of mutant ras proteins in myotubes alter the clustering of AChRs induced by agrin?

3) Is ras downstream of the MuSK signaling cascade induced by agrin?

The second part of this study focused on the potential interaction between the adaptor protein Shc and MuSK. MuSK and Trk intracellular tails have very homologous and Shc is an important downstream effector of Trk. In particular, both receptor tyrosine kinases share the Shc binding domain «NPXY» in their juxtamembrane domains. In this case, my aim was to find the answer to these questions:

1) Is Shc phosphorylated in tyrosine residues after agrin treatment?

2) Shc and MuSK interact together?

3) Is this interaction modulated by agrin?

Chapter 2

Materials and Methods

Material and Methods

Reagents

The following polyclonal antisera used in this study were: anti-MuSK (Carbonetto lab; for immunoprecipitation), anti-MuSK (from Michael Ferns, McGill University; for immunoblotting), anti-MEK1, anti-Grb2, anti-Shc and anti-Sos1/2 from Santa Cruz Biotechnologies (SCB), anti-H-ras from Oncogene, and anti-PI3K from Upstate Biotechnology Industries (UBI). The monoclonal antibodies used in this study were anti-Grb2, anti-phosphotyrosine PY20, anti-β-tubulin and anti-H-ras (SCB), anti-Shc and anti-phosphotyrosine RC20H (Transduction Laboratories), anti-phosphotyrosine 4G10 (UBI), anti-human-c-myc (Roche). Streptavidin agarose, most secondary antibodies coupled to fluorochromes as well as all alpha-Bungarotoxin products were from Molecular Probes. Secondary antibodies conjugated to HRP were from Jackson (goat anti-mouse-HRP and goat anti-rat-HRP) or Santa Cruz (goat anti-rabbit-HRP). PD098059 was obtained from Calbiochem (see below) and cycloheximide was from Sigma Chemicals. Dr Michael Ferns (McGill University) provided recombinant c-agrin$_{4,8}$. Cross-linkers DMS (dimethyl suberimidate) and DMP (dimethyl pimelimidate) were purchased from Pierce Co.. Bovine serum albumin (BSA) Fraction V and protease inhibitor cocktail were from Roche. Protein G agarose was from Gibco BRL. All other chemicals were from Sigma.

Tissue culture

The C2C12 cell line and a subclone of C2C12 over-expressing the CAR receptor (from Dr Paul Holland, MNI) are used. The cells were cultured on 100mm culture plastic plates (Falcon). The cells were plated at a density of $5x10^6$ cells per dish and grown at 37°C (8% CO_2) in Dulbecco's Modified Eagles Medium high glucose (DMEM-Hi) supplemented with 20% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin (P/S) and 0.5% Fungizone. At 70% confluence, they were subjected to DMEM supplemented with 4% horse serum, 1% P/S and 0.5% Fungizone, to induce myoblast fusion into myotubes. Fresh growth/fusion medium was administered daily. Splitting was done when the myoblasts reached 60% confluence. To do so, the dishes were washed

twice with Ca^{2+}/Mg^{2+}-free phosphate buffer saline (PBS) followed by a 2-4 minutes incubation at 37°C with 0.1% trypsin. All media were from Gibco BRL.

Recombinant adenoviruses and cell infection

Replication-defective recombinant adenoviruses expressing the ras mutants of interest (ras-N17, ras-V12, ras-V12-D38, ras-V12-T35, ras-V12-Y40; for nomenclature explanation, see table 1 in Chapter 3) were generously provided by Dr David Kaplan (Mazzoni et al., 1999). All ras mutants were tagged with a human myc epitope. Some of the adenoviral vectors also encoded for GFP as a reporter. Briefly, these adenoviruses were engineered to express a single bi-cistronic mRNA encoding two distinct proteins due to the presence of internal ribosomal entry sites (IRES). A similar adenovirus encoding only for GFP was used as a control (Quantum). All of the constructs were under the control of the cytomegalovirus-5 (CMV-5) promoter. C2C12-CAR myotubes were infected with the recombinant adenoviruses by incubating them with the appropriate amount of virus in a small volume for 2-4 hours to facilitate adherence to the cell surface receptors. Thereafter the cells were flooded with normal amount of medium. Typically, the cells were assayed 48 hours post-infection. Infection efficiency was assessed using an inverted fluorescence microscope (Zeiss) using GFP as an indicator (when available). Given that myotubes are multinucleated, it was difficult to determine the exact number of cells to be infected. To get a relative idea of the MOI (multiplicity of infection) to be used, I counted the numbers of cells 1 day after the change in fusion medium (it is known that myoblasts go through one additional round of cell division before starting to differentiate). Assuming that the number of cells incorporated into myotubes remained constant, I determined that an MOI of 25 to 50 could efficiently transduce the cells. Typically, 50 MOI was used for all viruses except for Adv-GFP (10 MOI) and Adv-ras-V12 (25 MOI).

Myotube treatments and protein extraction

Typically, 3 day old fused myotubes are treated with 1nM of recombinant $agrin_{4,8}$ for various lengths of time. Alternatively, the cells were exposed to various concentrations of ARIA or BDNF. After a careful wash with PBS, cells were scraped of the dish,

transferred into individual conical tubes with 1ml of protein extraction buffer (25mM Tris (pH7.5), 25mM glycine, 150mM NaCl, 5mM EDTA, 1% triton X-100, 1X protease inhibitor cocktail, 1mM $NaVO_3$ and 50mM NaF) for 20 minutes. Alternatively, the cells were extracted with RIPA buffer (25mM Tris pH7.5, 150mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, protease inhibitor cocktail, 1mM $NaVO_3$, 50mM NaF) for 1-2 minutes. Samples were spun for 5 minutes at 1000g and the pellet was discarded. All steps were carried out at 4°C. In some experiments, the pellet of Triton X-100 extracted cells was re-extracted using RIPA buffer to solubilize proteins associated with the cytoskeleton (Schlaepfer et al., 1998). Protein concentration was determined using the BioRad protein determination kit and was determined using a standard curve generated with bovine serum albumin (BSA). Reducing sample buffer (conaining SDS, DTT, Tris and glycerol) was added to the samples which were then heated at 97°C for 5 minutes. Samples were usually processed on the same day by SDS-PAGE. If not, 10% glycerol is added to the samples before they were snap-frozen in liquid nitrogen; samples were stored at -20°C for no more than 1 week.

Drug treatments

Various drugs were used to study their effects on AChR clustering or level on the membrane. These include a MEK inhibitor (PD098059) and a protein synthesis inhibitor (cycloheximide). All were diluted in DMSO and added to the medium for various length of time (as indicated). The optimal concentrations were determined so as to minimize cytotoxicity. Typically, the cells were processed 24 or 48 hours after drug exposure. As a control, an equal volume of vehicle (DMSO) is added to identical cultures; the amount of DMSO added was never more than 0.5% of the final volume of cell culture medium.

Immunoprecipitation

Immunoprecipitations (IP) were carried out by adding the relevant primary antibodies to the freshly thawed samples and mixed on a rocking platform for at least one hour at 4°C. Subsequently, 35ml of protein G agarose beads (pre-washed in IP wash buffer ; see below) is added to capture the protein-antibody complexes. The samples are

then spun at 4000rpm for 60 sec to pellet down the protein-G agarose beads and washed four times with IP wash buffer (50mM Tris (pH8.0) and 1% Triton X-100) with either low (150mM) or high (500mM) NaCl. Finally, reducing sample buffer is added to the pelleted beads, which were vortexed and heated at 97°C for 5 minutes. As a control, the beads alone are always used to assess non-specific binding.

Co-immunoprecipitation experiments were used to assess direct protein-protein interactions between MuSK and downstream effectors. Briefly, MuSK was immunoprecipitated from cells extracted with Triton X-100-based buffer as described above. The antibody bead/complexes were washed with buffer of different salt concentrations in order to evaluate the strength of the interaction. After SDS-PAGE and electroblotting, the filters were probed with appropriate antibodies to see if candidate molecules could be brought down with MuSK. Since I was unable to purify the MuSK anti-serum without losing all potent antibodies, I had to use the crude serum to perform immunoprecipitations. This rendered analysis in the region 45-60kD problematic since the heavy chains (from the MuSK anti-serum) were strongly cross-reactive, even with secondary antibodies that do not recognize rabbit IgG. To circumvent this problem, I cross-linked the anti-MuSK antiserum to protein-G agarose beads prior to IP using the cross-linkers DMS or DMP, essentially as described in Schneider et al. (1982). Briefly, the agarose beads were washed once with NaBr buffer (0.1M, pH8.2), followed by addition of the antiserum in the same buffer. After rocking for 30-60 minutes at room temperature, the tubes were gently spun to gather the pellet at the bottom. The agarose beads-antibody complex was washed twice more with NaBr buffer. Then, after one wash with triethanolamine buffer (0.2M, pH8.2), the cross-linkers were added (0.04M) diluted in triethanolamine buffer and the tubes were rocked for 60 minutes. The reaction was stopped by pelleting the complex, followed by resuspension in an equal volume of ethanolamine (0.04M, pH8.2). After 5 minutes of rocking, the complexes were washed three more times with NaBr buffer (pH8.2) and stored in NaBr buffer supplemented with 0.2% sodium azide. The efficiency of the cross-linking procedure was assessed by processing the samples from different steps of the procedure by gel electrophoresis 10%): the IgG band was easily visualized by Coomassie blue staining.

SDS-PAGE and Western blot analysis

The samples are loaded into the wells of a SDS-PAGE gel and electrophoresed as specified by manufacturer (BioRad) with a buffer containing 0.25M Tris, 0.2M glycine and 1mM SDS. For SDS-PAGE, the stacking gel was made with stock solutions of Tris (0.5M, pH6.8), 10% SDS, 30% acrylamide/Bis, 10% ammonium persulfate (APS) and TEMED. The separating gel was made with the same solutions except for Tris (1.5M, pH8.8). Pre-stained and unstained broad range molecular weight markers were from New England Biolab (NEB). The samples were electroblotted (100mV, 10mA) onto supported nitrocellulose membrane (Schleicher & Schluell) in a buffer containing 20% methanol, 0.25M Tris and 0.2M glycine. The nitrocellulose filters were blocked by for 1-2 hours incubation in a solution containing 5% BSA (fraction V), 150mM NaCl, 10mM Tris (pH7.5) and 0.1% Tween 20-20. Subsequently the appropriate primary antibody was diluted in the blocking solution and applied to the filters for at least 2 hours at room temperature or overnight at 4°C. Then, the membranes are rinsed (4x15min) in wash buffer (150mM NaCl, 10mM Tris (pH7.5) and 0.1% Tween 20-20), incubated with appropriate secondary antibody conjugated to HRP (1 hr) and washed again. The signal was revealed by soaking the membranes into a solution of enhanced chemiluminescence system (ECL Plus, from NEN) for 30-60 seconds and immediately exposed to Kodak films (XB-1) for various length of time. Backprobing was done on stripped nitrocellulose filters by bathing the membrane into a stripping buffer (0.2M Glycine, 0.1% Tween 20-20, pH2.5) for 10 minutes; thereafter the membrane is treated as described above. Densitometric analysis was performed using a scanner and the NIH 1.6 imaging software (details appear in the figure legends).

Fluorescence microscopy and immunostaining

Confluent cells were washed 1-2 times with PBS at 37°C followed by incubation for 5-20 minutes in 2% paraformaldehyde (PFA). For intracellular proteins, the cells were incubated with permeabilization buffer (0.1-0.3% Triton X-100, 5% BSA in PBS) for 3-10 minutes. The cells were then blocked for at least one hour with blocking buffer (5% BSA

in PBS) followed by overnight incubation with primary antibody diluted in blocking buffer. The following morning, the cells were carefully washed (4 times 15 minutes) with PBS and incubated with appropriate secondary antibody coupled to a fluorochrome. One culture was always treated with the secondary antibody alone to assess the specificity of the signal observed. Once the staining procedure was completed, Immunofloure was applied to the cells (1 drop per $5cm^2$), followed by covering with a glass coverslip of appropriate size (Fischer). All steps were done in the dark.

AChR clustering assay

The addition of various amounts of recombinant c-terminal $agrin_{4,8}$ was used to induce acetylcholine receptor (AChR) clustering to the fusion medium. After 24 hours, AChR were localized by exposing the cells to rhodamine-labeled a-BTX for 1 hour at 37°C (2ng/mL diluted in fusion medium). The cells were fixed and processed as described above. AChR clusters were visualized with fluorescence microscopy. AChR clusters were counted on myotubes of medium diameter which spanned the entire field (400X) and which were first identified by phase-contrast microscopy. For experiments with the adenoviruses, the clusters were counted at 630X in order to make sure that the clusters were from a GFP-positive myofiber. A cluster was defined as a continuous patch of bright staining, irrespective of its size; small dots were not counted as clusters. The average number of clusters was obtained by counting at least 50 myotubes from different fields per experiments.

AChR levels on the membrane

The levels of AChR on the membrane of myotubes were determined using ^{125}I-a-BTX (Amersham). Typically, the cell cultures were incubated in triplicates for 1 hour with a small volume of fusion medium containing 5ng/mL of ^{125}I-a-BTX (between 2000Ci/mol). Prior to this, a few wells were exposed to 2000-fold higher amount of cold a-BTX (10mg/mL) to assess the levels of non-specific binding (estimated to be about 1% of the total signal). The plates were put back into the incubator and gently rocked to insure proper distribution of the α-BTX over the entire surface of the well. Thereafter, the cells

were washed 4 times with PBS. For initial studies, the cells were scraped off the wells using a rubber policeman but this method was found to generate a lot of variability because the cells were not entirely removed from the well (especially in 24-wells plate). I solved that problem by incubating the cells in a solution of divalent cation-free PBS/0.1% trypsin for 10-15 minutes. The relative amounts of ^{125}I-a-BTX bound to the membrane were determined using a gamma counter (% bound / 1 minute). In order for this assay to reflect accurately the entire pool of AChRs, the amount of ^{125}I-a-BTX added had to be saturating. I calculated that 5ng/mL was about 2-3 times saturation by comparing the counts from control cells with that of an aliquot containing the same volume of ^{125}I-a-BTX added to the cells (assuming that ^{125}I-a-BTX binding efficiency was maximal). Each value obtained was compared to every value from the appropriate controls: for example, each value obtained with one particular virus was compared to all the values of the non-infected cells. The final value was an average of all percentages obtained for a single type of treatment. Statistical significance was determined by ANOVA (Fischer's PLSD).

Chapter 3

A role for ras during acetylcholine receptor aggregation?

Introduction

Ras activity can be modulated by many receptor tyrosine kinases activated by extracellular ligands (Pawson, 1994). The functional specificity of a given ligand is affected by the modulatory components that act on ras at any given moment (e.g., GAP, GEF and GDI) as well as by the activation of different subsets of ras downstream effectors (PI3K, raf-1, ralGDS). Therefore, ras plays a central role in many cellular processes since it appears to be able to integrate a plethora of convergent signals (Wittinghofer and Herrmann, 1995).

Our understanding of the signaling mechanisms underlying NMJ formation is still in its infancy (Colledge and Froehner, 1998a; Wallace, 1996). In spite of intense efforts, the only solid evidence gathered so far is the identity of the inducer (agrin), the initiator (MuSK) and the mode of the signaling cascade (tyrosine kinase/phosphatase; Sanes and Lichtman, 1999). It has been suggested that proteins like Grb2 (Yang et al., 1995; Colledge and Froehner, 1998b) or src-like kinases (Swope and Huganir, 1994; Fuhrer and Hall, 1996) might be involved in the process since they interact with synaptic proteins such as dystroglycan and AChR, respectively.

Several indirect findings that support a role for ras at the NMJ have been reported. For example, rac, a known ras downstream effector, appears to play an important role downstream of agrin/MuSK (Weston, C. 1998 Neurosci. Soc. Abstr.). Furthermore, neurotrophins signaling via TrkB (BDNF and NT-4) can dampen the effects of agrin, possibly via ras, as it is an important downstream target of TrkB (Wells et al., 1999; Klesse and Parada, 1999). The high degree of homology between the kinase domains of MuSK and Trk also raises the possibility that ras might be a common downstream target (Valenzuela et al., 1995). Finally, ras is implicated in AChR mRNA transcription induced by ARIA/erbB signaling (Tansey et al., 1996).

Based on these facts, I decided to investigate the role of ras during agrin-induced NMJ formation. The hypothesis is that ras might, as in other systems, be a point of convergence for signaling pathways originating from the RTKs erbB, TrkB and/or MuSK. The results obtained suggest that ras may be involved in AChR clustering induced by

agrin, but do not allow me to draw firm conclusions on this issue. The significance of the results as well as improvements to the experimental paradigms is discussed.

Table 1 **The different ras mutants and the pathways that they activate**. Mutations in the ras proteins can affect dramatically its functions. Some mutations result in constitutive ras activity (ras-V12) while another one completely abrogate its function (ras-N17). There are three main ras downstream effectors (PI3K, raf1 and ralGDS). Point mutations inflicted to the switch region I of ras-V12 change considerably the signaling pathways that it utilizes.

	Downstream effectors		
	raf-1	PI3K	ralGDS
ras (WT)	+	+	+
ras-N17	-	-	-
ras-V12	+	+	+
ras-V12-D38E	+	-	-
ras-V12-Y40C	-	+	-
ras-V12-T35S	+	-	-

Results

In order to study the role of ras downstream of agrin/MuSK, I decided to infect cultured myotubes with adenoviruses (Adv) encoding different ras mutants (see table 1 and Material and Methods). Since ras is implicated in many signaling pathways such as those involved in myogenesis and AChR transcription, it was important to determine the optimal length of time between infection and agrin treatment that would insure proper mutant protein expression while minimizing collateral alterations that might confound the results (e.g. erbB signaling).

Adenovirus infection of C2C12 and C2C12-CAR cells

Initial attempts to infect fused C2C12 cells with Adv-GFP (green fluorescent protein) yielded very few GFP-positive myotubes but many myoblasts were intensely green (personal observation). Similar results were observed with both ras-N17 (dominant inhibitory) and ras-V12 (dominant positive); both adenoviruses also encoded GFP as a marker and were myc-tagged. I also observed that ras-V12 prevented myotube formation and led to uncontrolled cellular proliferation, as reported previously by Vaidya et al. (1991). Conceivably, the expression of ras-N17 might have the reverse effect: blockade of cell proliferation and promotion of myogenesis. Myoblasts expressing this mutant almost never fused into myotubes indicating that ras-N17 does not promote myogenesis. We did, however, observe that ras-N17 infected myoblasts (identified by GFP expression) were not proliferating. This showed that the two ras mutants had, at least in the context of myoblasts, distinct and opposite effects. Ras-N17 mutant did not appear to be cytotoxic for the myoblasts since neither vacuolation nor cell death was apparent. While the most plausible explanation was that ras-N17 stopped cellular proliferation, it was possible that the cells divided normally but did not share the epichromosome encoding ras-N17 contributed by the adenovirus. To address this issue, a dish of confluent myoblasts was split one day after adenovirus infection and plated into a new well; an identical dish of non-infected myoblasts was also split. Thereafter the growth of the two groups of cells was compared. It is noteworthy that ras-N17 myoblasts adhered to the dish normally,

indicating that inhibition of ras did not interfere significantly with substrate attachment. While the population of non-infected cells typically doubled after overnight incubation, the number of ras-N17-positive cells did not change, even after 3 days in culture, a time point at which the control chamber was already confluent. Daily visual examination confirmed that the GFP positive cells did not divide. As an additional proof, the rate of proliferation of cells infected with Adv-GFP was similar to that of uninfected cells (personal observation), indicating that neither the infection process, the adenoviral proteins nor GFP itself contributed to the effects observed with ras-N17. From these results, it is clear that C2C12 cells are not suitable to study the effects of ras mutants on agrin-induced AChR clustering since it was nearly impossible to infect myotubes directly or to get infected myoblasts to fuse into myotubes. Recently, it was demonstrated that the inability of adenovirus to infect myofibers is due to a dramatic downregulation of a membrane protein called CAR (Coxsackie-adenovirus receptor) that is required for virus internalization (Nalbantoglu et al., 1999). Fortunately, I was able to get a subclone of C2C12 cells engineered to overexpress CAR in both myoblasts and myotubes (from Dr Paul Holland, Montreal Neurological Institute). C2C12-CAR myotubes were very efficiently transduced with recombinant adenoviruses (see below).

In my hands, C2C12-CAR cells grew slightly slower than normal C2C12 and could be kept in fusion medium for extended periods (up to 12 days versus 3-4 days for C2C12). MuSK activation and AChR tyrosine phosphorylation in C2C12-CAR cells was not as strong as in C2C12 cells (figure 3.1 A and B), but the extent of agrin-induced AChR clustering did not appear to be significantly different when C2C12 and c2C12-CAR cells were treated with similar amounts of c-agrin$_{4,8}$ (personal observation). Comparison of developmental regulation of ras expression between C2C12 and C2C12-CAR cells showed a similar pattern of expression. Indeed, it was relatively low in myoblasts (G3) and early myotubes (F1) but increased on day 2 in fusion medium, about the time when myotubes were starting to be responsive to agrin (figure 3.2 A). Preferential expression of ras in myotubes was confirmed by immunocytochemistry with an anti-H-ras serum (data not shown). Expression of β-tubulin was used as a loading control to show that the differences in ras levels were not due to unequal protein amounts. Comparison of the

expression pattern of other signaling proteins known to interact with ras such as Sos, Shc and Grb2 showed no significant differences between the two cell lines, although the levels of p47Shc might be slightly reduced in C2C12-CAR cells relative to WT cells (figure 3.2 B). It is noteworthy that the blots presented in figure 3.2 B were generated from cellular extracts of fusion day 2 (F2). Based on these findings, it was concluded that C2C12-CAR cells are suitable for studying the effects of mutant proteins during synapse formation. Still, this cell line is not optimal for extensive biochemical studies given the low level of MuSK and AChR phosphorylation after agrin treatment. All subsequent experiments were done with the C2C12-CAR cells to facilitate comparisons.

Transgene expression

Indirect evidence of transgene expression was obtained in the myoblast study above since both ras-V12 and ras-N17 infected myoblasts behave as expected. Still, it was important to determine whether the mutant proteins of the different transgenes were expressed after adenoviral infection of C2C12-CAR myotubes. Whole-cell lysates from infected cells were first probed with an anti-H-ras serum; ras mutant proteins are easily identified since they have slightly higher molecular weights than the native ras proteins because they are myc-tagged (Mazzoni et al., 1999). As shown in figure 3.3 A, ras mutant proteins (N17 and V12) were expressed, albeit at different levels. Note that no band of similar molecular weight was observed in the Adv-GFP lane or in the uninfected one; the doublet at 21kD corresponds to farnesylated and unprocessed ras wild type proteins. Clearly, ras-V12 levels are much higher than any other transgene. Expression of ras-N17 and ras-V12 appeared to result in reduced levels of native ras proteins, but this might be a simple reflection of underloading (compare the β-tubulin levels). Certainly, the decrease was not as drastic as that observed after cycloheximide treatment, a protein synthesis inhibitor that does not seems to affect the concentration of β-tubulin. Unfortunately, I did not backprobe with an anti-c-myc antibody to be certain that the levels observed with the H-ras antibody were accurate.

All recombinant adenoviruses used in this study encoded a single mRNA that is translated into two distinct proteins (mutant ras and GFP) because of an internal ribosomal

entry site (IRES) located in the middle of the transcript. Therefore, another important control that was required before analyzing the effects of the mutant ras on AChR clustering was to determine whether GFP expression correlated with that of the transgenes, both in terms of intensity and localization. It was found that, in general, GFP intensity was lower in cells infected with ras-V12 when compared to that of ras-N17 or GFP alone. In contrast, immunocytochemical studies with anti-c-myc antibody revealed that ras-V12 infected cells expressed high levels of the transgene whereas the intensity of the myc staining for ras-N17 infected cells was much lower (figure 3.3 B and data not shown). Importantly, all green cells were positive for myc but the intensity of GFP did not correlate perfectly with that of myc staining. Furthermore, some ras-V12 expressing cells that were clearly GFP negative were myc positive. Hence, all GFP-positive cells are myc-positive but the inverse is not necessarily true. Finally, in most experiments, Adv-ras-N17, Adv-ras-V12, Adv-ras-V12-D38 and Adv-GFP infected approximately 75% of the myotubes (personal observation). From these results, I concluded that counting AChR clusters on GFP positive cells would provide a clear picture of the effect triggered by of these various mutants.

AChR levels on the membrane

In order to analyze the effects of the various ras mutants on AChR clustering, it was important to verify the levels of AChR on the membrane since ras plays a central role in the induction of AChR transcription by ARIA (acetylcholine receptor inducing activity). Furthermore, muscle ARIAis expressed by C2C12 cells and may control the levels of AChR in the absence of neural ARIA. ^{125}I-α-BTX was used to label AChR because it is impermeant to the cell, thus giving values that reflect accurately the levels of AChR at the plasma membrane. All assays were done 48 hours post-infection. Unexpectedly, expression of GFP alone yielded values that were slightly but consistently lower than the uninfected cells ($p<0.05$; figure 3.4). Ras-V12 scored higher than control but it appears unlikely that such a modest increase (vs. control) would account for the increase in spontaneous AChR reported above. The myotubes in these studies did not display extensive vacuolation. Preliminary results with mutants ras-V12-Y40 and ras-V12-T35

displayed no change in AChR levels. It was impossible to assess the efficiency of the adenoviral infection since none of them co-expressed GFP and it is possible that these results simply reflects the fact that no mutant proteins were expressed. Consequently, these data should not be regarded as truly indicative of the effects these mutants may have on AChR levels. A more thorough study of the effects of these mutants on AChR clustering was not possible due to limited supply of adenovirus. Ras-V12-D38 and ras-N17 infected myotubes were not significantly different than controls. The lack of significant differences in AChR number between all treatments and the controls indicated that effects observed with these ras mutants were not due to major alterations in the levels of AChR at the membrane.

To determine if the inhibition of the ras pathway by ras-N17 resulted in a decrease in AChR levels, a pharmacological agent, namely PD098059, was used to inhibit the function of MEK, a protein known to be involved in AChR transcription downstream of ras. To do so, the cells were incubated for 48 hours with various concentrations of PD098059. As shown in figure 3.5, a dose-dependent reduction in AChR on the membrane was observed with PD098059. Assuming nearly complete MEK inhibition at a concentration of 100μM, these data indicate that MEK may regulate no more than 50% of the AChR protein pool. It is noteworthy that no extensive cell death was observed after drug treatments at all concentrations used (personal observation).

AChR clustering

To study the effects of the ras mutants on AChR clustering, fully fused C2C12-CAR myotubes were infected. These were subjected to overnight agrin treatment 24 hours post-infection, i.e. when the levels of GFP and transgene were maximal. In this study, the effects of the mutants were compared to non-infected cells or to myotubes infected with Adv-GFP, which had no effect, by itself, on AChR clustering induced by c-agrin$_{4,8}$. It was found that infection with ras-V12 often resulted in obvious vacuolation and cytotoxicity 48 hours post-infection, and such cells were not very responsive to agrin (personal observation). Since a similar pattern was observed in uninfected C2C12-CAR cells cultured for 12-15 days (as opposed to 3-5 days for ras-V12 infected myotubes), it is

possible that expression of ras-V12 accelerated the aging process, possibly by inducing an apoptotic pathway (this avenue was not further investigated). In ras-V12 infected cells that did not display extensive vacuolations, possibly because of lower transgene expression, AChR clustering was observed, even in the absence of exogenous c-agrin$_{4,8}$ (figure 3.6). Preliminary results indicates that infection of myotubes with ras-V12 does not alter the effects of maximal c-agrin$_{4,8}$ (100pM) since the levels of AChR clustering in ras-V12 myotubes were similar to that of GFP infected or non-infected myotubes (data not shown). Unfortunately, confirmation of these data is lacking because in the time interval between cell fixation and quantification, the quality of the cells deteriorated. It is important to note that this study was not conducted with submaximal agrin concentration (<100pM), thereby potentially masking the additive effects of ras-V12 and agrin. Time constraints did not allow me to repeat these crucial experiments.

Studies with ras-V12-D38, which activates only raf-1 because of a point mutation in the effector domain (see table 1), were informative because it did not cause the extensive vacuolation observed with ras-V12. This might reflect the fact that it was expressed at lower levels than ras-V12 (figure 3.3 A), or that ras signaling via downstream effectors other than raf-1 promote cell death. Interestingly, expression of ras-V12-D38 also yielded an increase in agrin-independent AChR clusters (figure 3.6 and personal observation). This preliminary result indicates that the positive effects mediated by ras during AChR clustering might be mostly conveyed via raf-1. This experiment should have been repeated a few more times for the analysis to reach statistical significance. This conclusion would have been strengthened if experiments conducted with ras-V12-Y40, which activates PI3K but not raf-1, did not promote AChR clustering. Unfortunately, the adenovirus encoding for ras-V12-Y40 did not co-express GFP rendering analysis much less efficient than with the other vectors.

Expression of dominant-inhibitory ras mutant (ras-N17) in myotubes did not cause any major sign of cytotoxicity indicating that blockade of H-ras function is not deleterious to critical cellular functions. Ras-N17 did not alter significantly the level of spontaneous clusters but reduced the impact of agrin addition on the formation of large AChR clusters (figure 3.6). Only large AChR aggregates displayed on medium size myotubes were

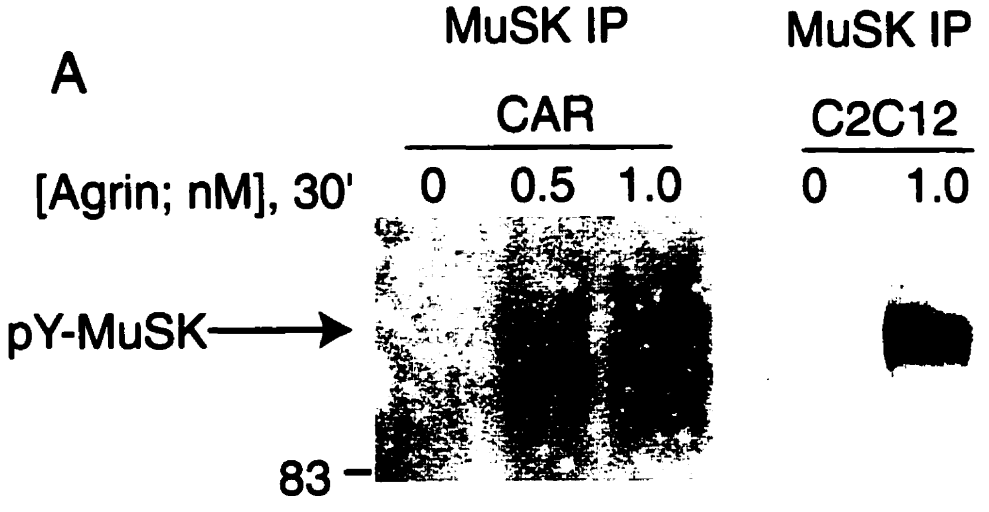
quantified. It is noteworthy that many myotubes, especially the larger size ones, were decorated with arrays of small clusters (figure 3.6; personal observation). These punctate clusters were not included in the data analysis because AChR clusters were defined as large continuous patches of rhodamine-labeled α-BTX.

Since incubation of myotubes with the MEK inhibitor PD098059 reduced the number of AChR levels on the membrane, I was interested to investigate the effects of MEK inhibition on agrin-induced AChR clustering. The cells were pre-incubated with the drug for 1 hour before addition of 20-100pM agrin to insure that MEK inhibition was effective before initiation of agrin treatment. The concentration of PD098059 used (5μM) was expected to have minimal effects on AChR levels (see figure 3.5). Addition of the drug had no effect on spontaneous AChR clustering when applied alone, and it did not result in significant changes when applied in combination with optimal c-agrin$_{4,8}$ concentration (100pM). In contrast, PD098059 potentiated the clustering effects of submaximal c-agrin$_{4,8}$ (20pM), reaching approximately the same level of AChR clustering as that observed with 100pM c-agrin$_{4,8}$ (figure 3.7). Furthermore, after treatment with both 20pM c-agrin$_{4,8}$ and 5μM PD098059, the percentage of myotubes bearing no AChR clusters was similar to that of myotubes exposed to 100pM c-agrin$_{4,8}$ (6%); the percentage of myotubes with AChR clusters on myotubes treated with 20pM c-agrin$_{4,8}$ alone was 30%. Therefore, PD098059 can potentiate the effects of submaximal c-agrin$_{4,8}$, indicating that agrin may relieve inhibitory components, possibly MEK, to induce AChR clustering.

Ras downstream of MuSK?

One way to determine if ras is involved in agrin/MuSK is to compare the phosphorylation levels of MuSK and AChR β-subunit, with or without agrin. Two attempts failed at determining whether expression of the ras mutants had any effects on these posttranslational modifications (data not shown). These results were inconclusive because I observed neither MuSK nor AChR tyrosine phosphorylation in both uninfected and infected cells. Since small chambers were used for adenoviral infection (an area about ten times smaller than the culture dish commonly used for biochemical analysis), these

negative results probably emphasize the fact that the immunoprecipitation (for MuSK) and the α-BTX precipitation (for AChR) were unsuccessful because of the low amount of proteins used. These inconsistent results were presumably neither caused by a decline in the health of the cells nor by a lack of response to agrin since AChR clustering proceeded normally.


A
MuSK IP
MuSK IP
CAR
C2C12
[Agrin; nM], 30'
0
0.5
1.0
0
1.0
pY-MuSK
83

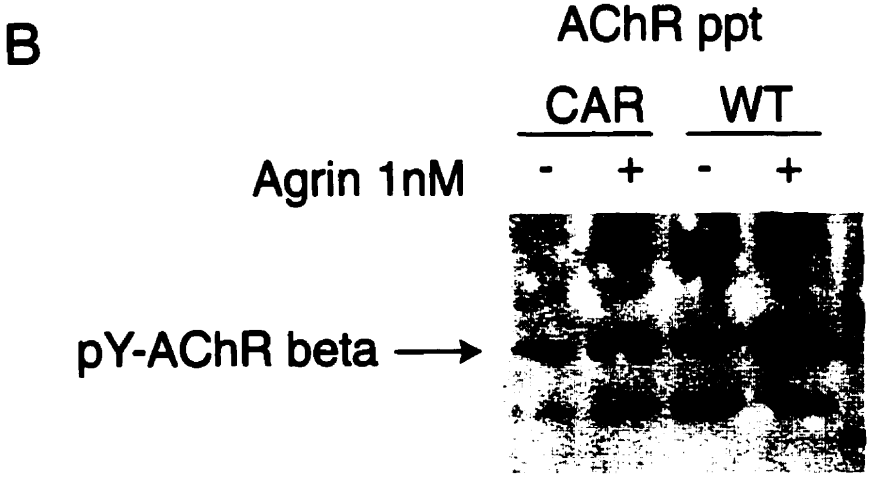
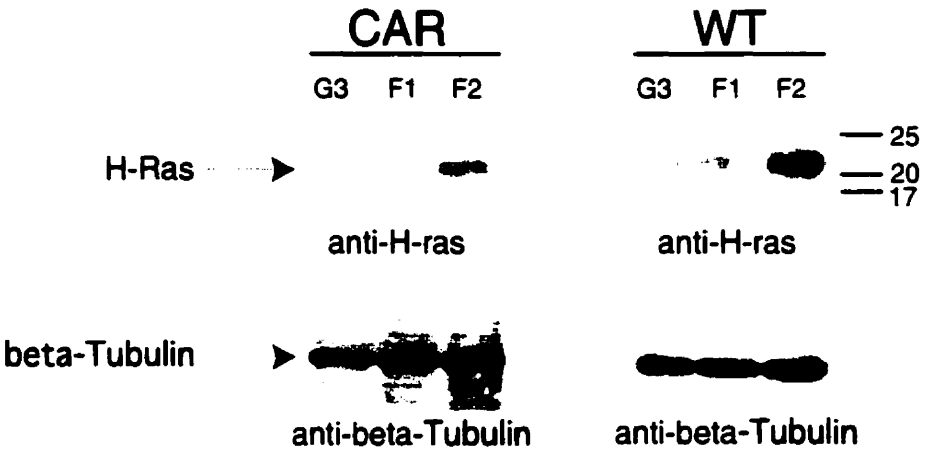

B
AChR ppt
CAR
WT
Agrin 1nM
-
+
-
+
pY-AChR beta

Figure 3.1 A **MuSK activation by agrin in C2C12-CAR and C2C12 cells**. C2C12-CAR and C2C12 myotubes were incubated with the indicated concentrations of agrin (0, 0.5 or 1nM) for 30 minutes. After two washes with ice cold PBS, the cells were extracted with a Triton X-100 buffer and MuSK was immunoprecipitated from the cell lysates using a polyclonal anti-MuSK antiserum. After SDS-PAGE (8%) and western blotting, the activation of MuSK was determined by immunoblotting with a monoclonal anti-phosphorytosine antibody (4G10). The C2C12 immunoblot is presented to facilitate comparison.

Figure 3.1 B **Acetylcholine receptors β-subunit phosphorylation in response to agrin in C2C12-CAR and C2C12 cells**. Dishes containing fuly differentiated C2C12-CAR and C2C12 cells were exposed to 1nM of c-agrin$_{4,8}$ for 30 minutes. Simultaneously, the cells were also incubated with biotinylated α-Bungarotoxin. After 30 minutes, the cells were carefully washed with ice cold PBS (2X) and extracted with a Triton X-100 buffer. The cell lysates were then incubated with streptavidin-agarose beads at 4°C to precipitate the AChR bound to α-Bungarotoxin. The samples were then subjected to SDS-PAGE (10%) and electrotransfered onto a nitroclellulose filter. Immunoblot analysis was done with antiphosphotyrosine antibodies (a mixture of both 4G10 and PY20).

A


CAR
WT
G3 F1 F2
G3 F1 F2
H-Ras
25
20
17
anti-H-ras
anti-H-ras
beta-Tubulin
anti-beta-Tubulin
anti-beta-Tubulin

B

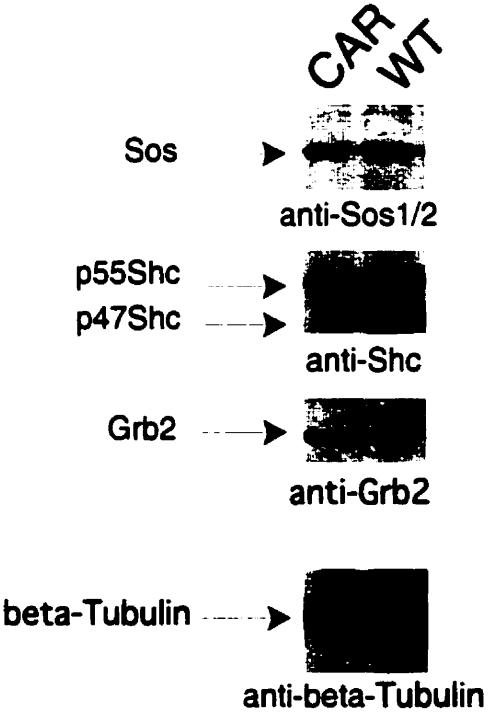
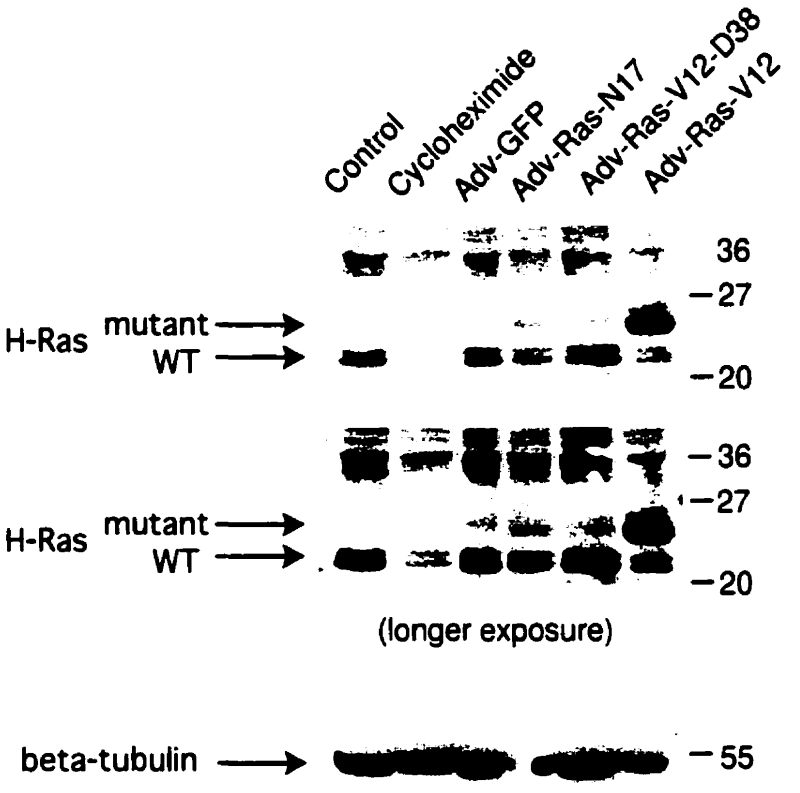

CAR
WT
Sos
anti-Sos1/2
p55Shc
p47Shc
anti-Shc
Grb2
anti-Grb2
beta-Tubulin
anti-beta-Tubulin

Figure 3.2 A **Developmental regulation of ras expression in CAR and C2C12 cells**. C2C12-CAR and C2C12 cells were extracted at different time point (G3, growth medium day 3; F1, fusion medium day 1) with a Triton X-100 buffer. Reducing sample buffer (3X) was added to the cell lysates prior to processing by SDS-PAGE (15%) and western blotting. Protein content was equalized using the reagent of BioRad. Immunoblotting of was done with anti-H-ras monoclonal antibody followed by an anti-mouse-HRP secondary antibody; the signal was revealed with enhanced chemiluminescence Plus system. The differences observed were not due to differences in loading since the levels of β-tubulin were similar in each lanes.

Figure 3.2 B **C2C12-CAR and C2C12 myotubes express similar levels of signaling proteins**. Cell lysates were obtained from both cell types and processed exactly as described in figure 3.2 A. The only difference is that the gel for Sos and β-tubulin was 8% bis-acrylamide whereas the one for Shc and Grb2 12%. Immunoblot analysis was done with anti-Sos, anti-Shc, anti-β-tubulin and anti-Grb2 antibodies followed by anti-rabbit-HRP or anti-mouse-HRP.

A


Control
Cycloheximide
Adv-GFP
Adv-Ras-N17
Adv-Ras-V12-D38
Adv-Ras-V12
H-Ras mutant
WT
36
27
20
H-Ras mutant
WT
36
27
20
(longer exposure)
beta-tubulin
55

B


GFP
c-myc
Ras-V12

Figure 3.3 A **Ras mutant proteins are expressed after infection.** Differentiated C2C12-CAR cells were infected with various recombinant adenoviruses as specified in the Material and Methods. Extraction was performed 48 hours post-infection. Samples were processed by SDS-PAGE (15%) and electroblotted on nitrocellulose filter, followed by immunoblotting with monoclonal anti-H-ras. Wild type ras is a doublet while the mutant forms runs at slightly higher molecular weight due to the myc tag. Probing with anti-β-tubulin indicated that the loading were approximately equal except for ras-N17. The high level of ras-V12 expression was confirmed in another independent experiment.

Figure 3.3 B **GFP is a valid marker for localization of ras mutant proteins but not for expression levels.** Two days post-infection, myotubes infected with ras-V12 were fixed with paraformaldehyde (2%; 10 minutes), permeabilized for 3 minutes with Triton 0.1%, and probed overnight with an anti-c-myc monoclonal antibody (5μg/mL). The signal was revealed using a secondary antibody conjugated to rhodamine. The specificity of the signal was verified by incubating a parallel well with the secondary antibody alone in each case. GFP stands for green fluorescent protein. Ras-N17 myotubes were treated similarly and displayed a similar pattern of staining that was weaker in intensity (data not shown).

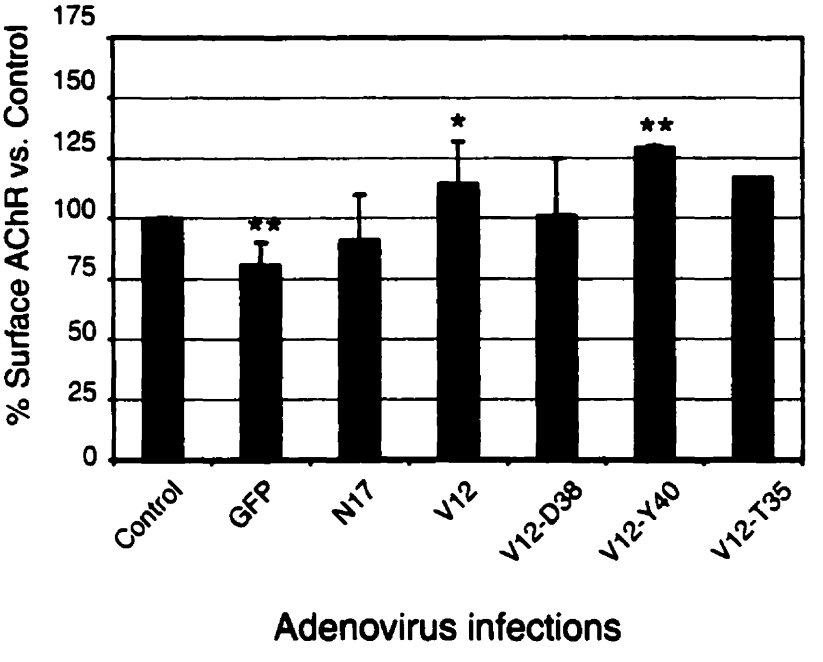

% Surface AChR vs. Control
175
150
125
100
75
50
25
0
**
*
**
Control
GFP
N17
V12
V12-D38
V12-Y40
V12-T35
Adenovirus infections

Figure 3.4 **Expression of various ras mutants in myotubes can alter AChR levels at the membrane**. Fully differentiated C2C12-CAR were infected with various recombinant adenoviruses as described before. After two days, the levels of membrane AChR were determined by adding 5ng/mL ^{125}I-α-bungarotoxin for 60 minutes. After many washes with PBS, the cells were split with trypsyn and transfered into plastic tubes to be counted with a gamma counter. The percentage of bound radioactivity was measured for 1 minute. Cold α-bungarotoxin (10μg/mL) was added to some wells to assess the level of non-specific ^{125}I-α-bungarotoxin binding: it was found to be less than 1% of total counts. Most experiments were done in triplicates (one was done in duplicate). Calculations: each value obtained was divided by all three values of the appropriate controls to determine the degree of change in percentage; all the percentages for a given treatment were averaged. This graph presents the percentage of change in membrane AChR levels triggered by expression of various ras mutants relative to uninfected cells. The data presented is the mean +/- SEM from different experiments; n=4 for ras-V12 and ras-N17, n=3 for GFP and ras-D38, n=2 for Ras-Y40C, and n=1 for ras-V12-T35S. ** indicates $p<0.005$ and *, $p\leq0.05$ by ANOVA Fischer's PLSD.

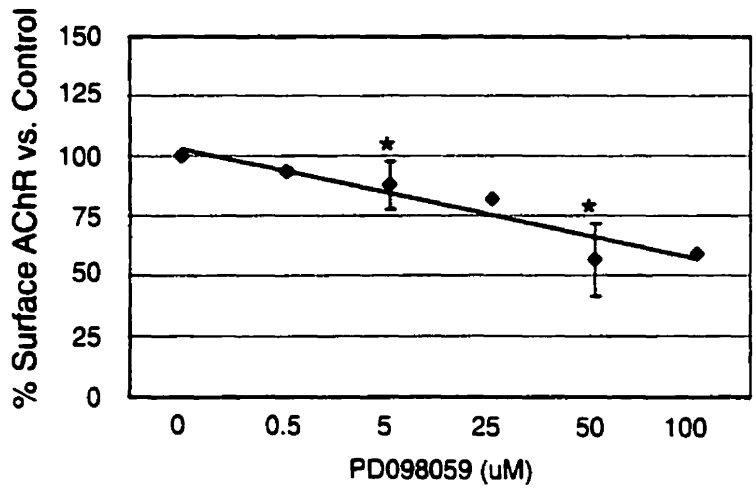
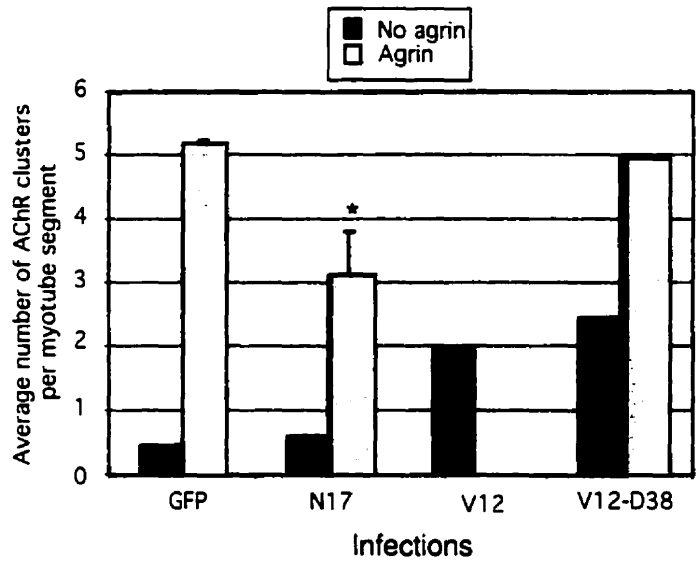

% Surface AChR vs. Control
150
125
100
75
50
25
0
0
0.5
5
25
50
100
PD098059 (uM)
*
*

Figure 3.5 **MEK inhibition causes a decrease of membrane AChR.** Differentiated C2C12-CAR cells were exposed for 48 hours to various concentrations of MEK inhibitor PD098059. This compound was diluted in DMSO and the latter was use as a control (zero point); DMSO had no effect on AChR receptor levels (data not shown). AChR levels were assessed using ^{125}I-α-bungarotoxin as described previously. Values are mean +/- SEM. For 0.5, 5 and 50μM, n=4; for 25 and 100μM, n=1. * indicates $p<0.005$ by ANOVA Fischer's PLSD test.

A


No agrin
Agrin
Average number of AChR clusters per myotube segment
0
1
2
3
4
5
6
*
GFP
N17
V12
V12-D38
Infections

B

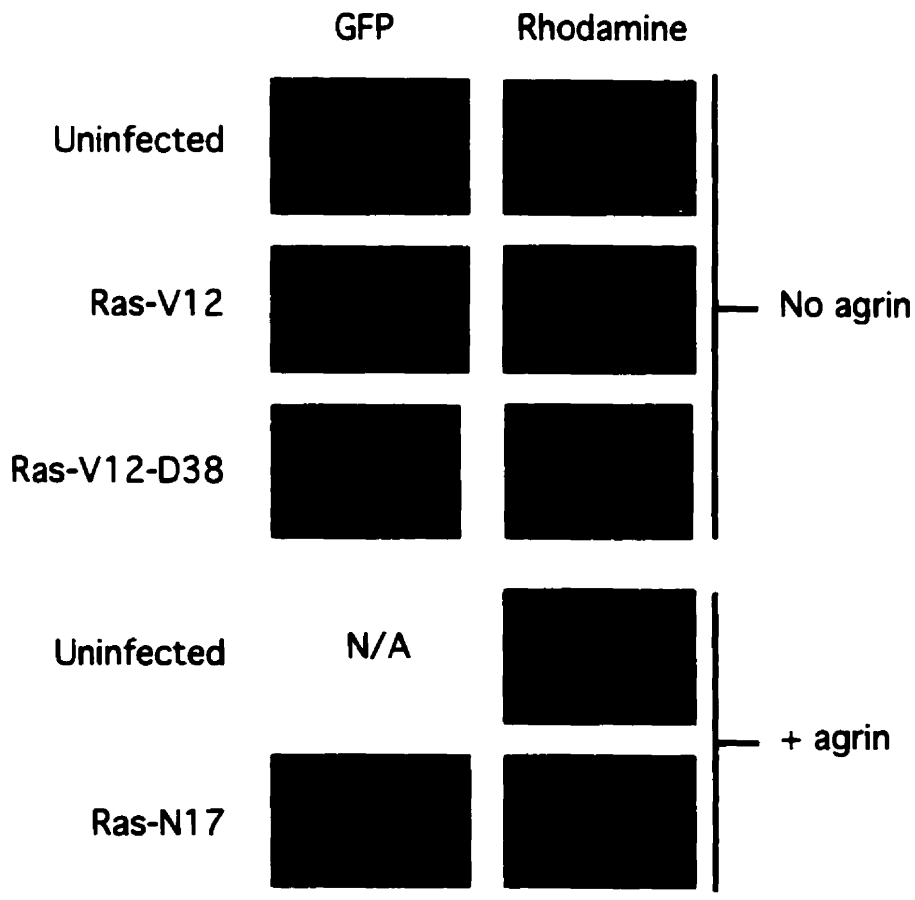

GFP
Rhodamine
Uninfected
Ras-V12
Ras-V12-D38
No agrin
Uninfected
N/A
Ras-N17
+ agrin

Figure 3.6 **Expressing ras mutants in myotubes impacts on AChR clustering**. One day post-infection, myotubes were incubated overnight with or without 100pM agrin to induce AChR clustering. The following day (48 hrs post-infection), rhodamine-labeled α-bungarotoxin was added to the medium for 60 minutes to stain surface AChR. The cells were then processed for fluorescence microscopy as indicated in Material and Methods. AChR clusters on GFP-positive myotubes of medium sizes were counted. The values presented are averages of 75 myotube segments from one experiment for ras-V12 and ras-V12-D38 and from two independent experiments for ras-N17 and GFP. *Top*, average numbers of AChR clusters per myotube segment. *Bottom*, Representative images of the different treatments.

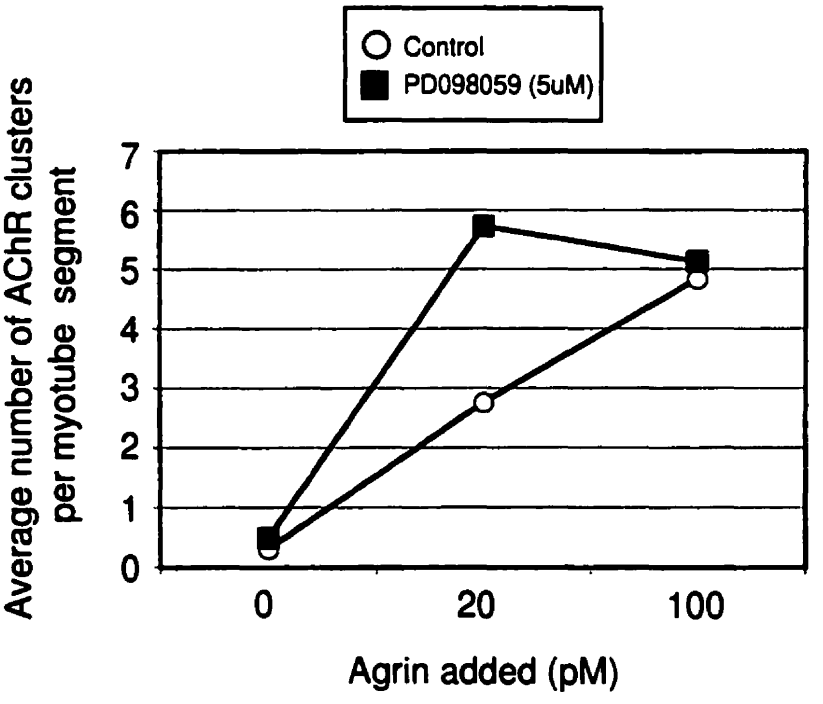

Control
PD098059 (5uM)
Average number of AChR clusters per myotube segment
0
1
2
3
4
5
6
7
0
20
100
Agrin added (pM)

Figure 3.7 **MEK inhibition potentiates the clustering effects of submaximal agrin**. The cells were incubated with 5μM of PD098059 for 1 hour before addition of agrin (20pM or 100pM). The following day, the cells were stained with rhodamine-labeled α-bungarotoxin and the AChR clusters were counted as mentioned before (except that the counting was done at 40X). Values are averages of 50 myotubes segments from one experiment. Similar results were obtained in another independent experiment.

Discussion

At the neuromuscular junction, ras plays an important role in the induction of AChR transcription by transmitting the signals emitted by ARIA/erbB to the nucleus (Tansey et al., 1996). No direct evidence have been put forward for a role for ras in agrin-induced clustering *per se*. Since ARIA has no effect on AChR clustering (Falls et al., 1993), the possibility that ras might be important during the process of AChR aggregation may seem counterintuitive. However, this study provides preliminary evidence suggesting that ras might also modulate the clustering of AChR induced by agrin. Figure 3.8 is a schematic summary of the data presented in this study.

Effects of PD098059 and ras mutants on AChR levels

In experiments on the effects of the various ras mutants on AChR levels, the expression of none of the mutants resulted in significant changes when compared to control (figure 3.4). Although the general trend of the ras-V12 mutants (in particular, ras-V12, ras-V12-Y40 and ras-V12-T35) suggests they might increase slightly AChR levels, it is unlikely that such differences would effect AChR clustering.

The fact that no ras mutants had significant effects on AChR protein levels at the membrane is somewhat surprising given that it was found that the same mutants (ras-N17 and ras-V12), when transfected and expressed under the control of a muscle-specific promoter, could affect the induction of AChR mRNA transcription (Tansey et al., 1996). In effect, ras-N17 efficiently blocked the increase in mRNA triggered by ARIA while ras-V12 bypassed the ARIA requirement. Based on this study, we can predict that expression of ras-V12 in myotubes should affect endogenous AChR mRNA transcription, but nothing can be inferred with regard to ras-N17 on this issue. While a significant increase (or decrease) in AChR mRNA levels may be important physiologically, it is important to stress that it does not necessarily cause a rapid increase in protein levels. Post-transcriptional negative feedback loops may also regulate the amount of protein that is being produced. Evidently, the present study would have been strengthened if an analysis of AChR mRNA would have been carried out to see if these results were reproducible. Another important

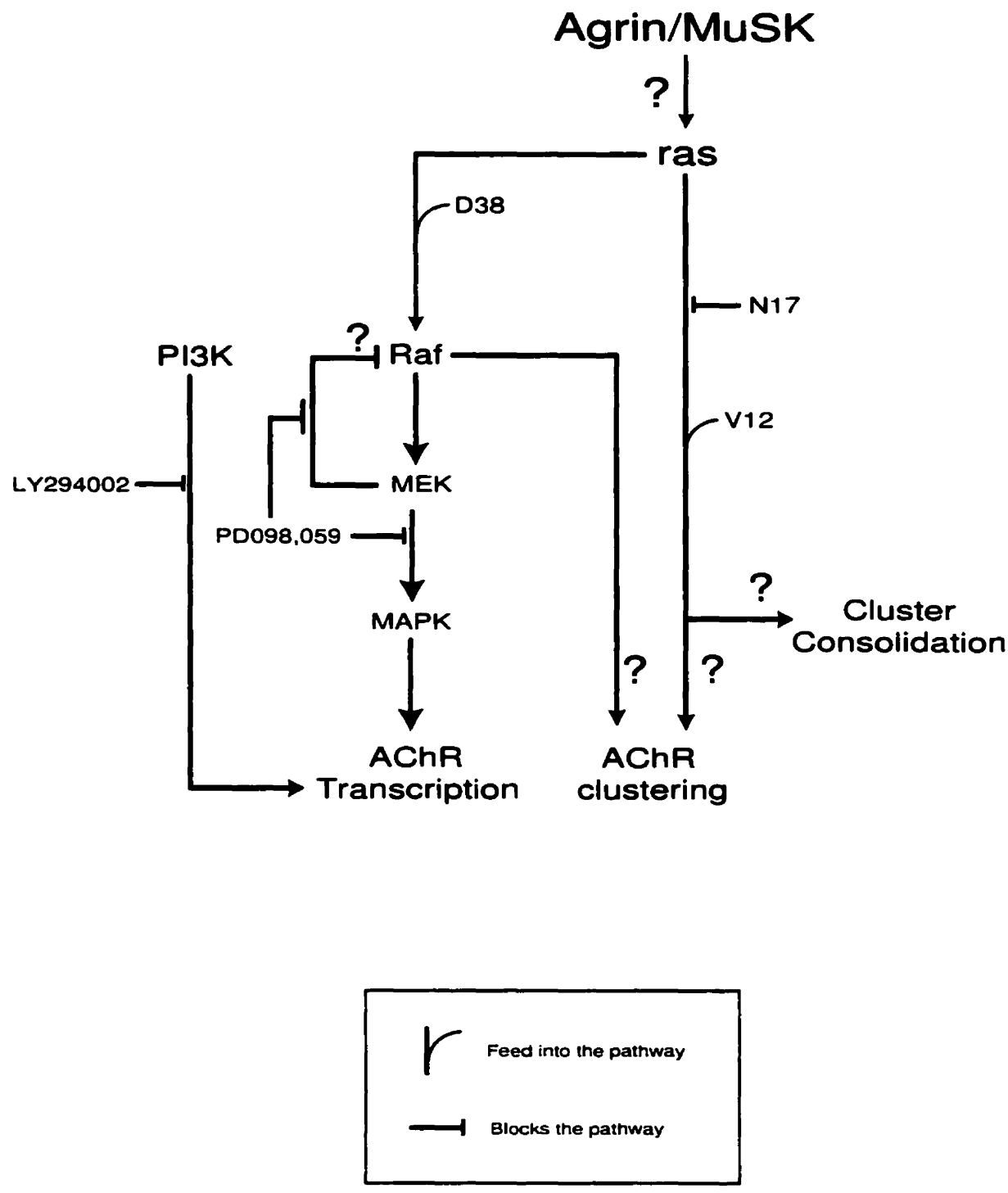

Agrin/MuSK
?
ras
D38
N17
?
PI3K
Raf
V12
LY294002
MEK
PD098,059
MAPK
?
Cluster
Consolidation
?
?
AChR
Transcription
AChR
clustering
Feed into the pathway
Blocks the pathway

Figure 3.8 **Ras, raf-1 and MEK may play a role during AChR clustering.** This model incorporates the results presented in this study to what is already published relative to ras at the NMJ. The pathway onto which ras-N17 and ras-V12 feed into is general as it concerns all possible ras downstream effectors. The pathway activated by ras-V12-D38 activates only raf-1. Note that an inhibitory signal from MEK to raf-1 was presented in figure 1.3.

control to verify the extent of ras-N17 inhibition would have been to expose myotubes to ARIA and look whether the increase in AChR mRNA was inhibited.

It is possible that the discrepancies observed between my results and those published by Tansey et al. (1996) may reflect the fact that the length of time (48 hours) was insufficient to see significant differences at the protein level. Alternatively, these contradictory results may reflect differences in the expression level of the ras mutant proteins caused by the different mode of transgene transfer (transfection vs. adenoviral infection) as well as by the distinct activities of the promoters involved. Supporting this view is the fact that protein levels correlate with the degree of myogenesis inhibition by ras mutants (Weyman et al., 1997). Finally, the apparent lack of effect on ras mutants on AChR levels may stem from the fact that the percentage of infected myotubes was overestimated. If so, the differences in AChR levels would masked the high percentage of normal, uninfected myotubes that bias the result towards normality. Such problem was circumvented by Tansey et al. (1996) by using lacZ reporter gene under the control of the AChR epsilon promoter, which allowed direct detection of positive myotubes.

As mentioned above, it would have been expected that inhibition of ras activity by ras-N17 would not only affect AChR mRNA, but also the proteins at the membrane. In order to determine whether the lack of effect of ras-N17 was due to the low levels of mutant protein expressed, a pharmacological agent (PD098059) was used to inhibit the activity of MEK, a major signaling protein downstream of ras/raf-1 pathway (Marais and Marshall, 1996). The results obtained with PD089059 give reason to think that the inhibitory effects of ras-N17 were incomplete (figure 3.5). Indeed, assuming that the effects of PD098059 were specific, the significant decrease in AChR levels could be attributable to inhibition of MEK only. Since MEK is located downstream of ras/raf-1 signaling, one would expect that inhibition of either ras or MEK may lead to similar, but not entirely overlapping cellular consequences. For example, expression of ras-N17 and inhibition of MEK by PD098059 both reduced the ability of ARIA to induce AChR mRNA transcription in chicken myotubes (Tansey et al., 1996). Thus, it is likely that the conjugation of low transgene expression with a lower percentage of myotubes infected may have hidden the actual effects of the mutants. To address this issue, radioactively

labeled α-BTX followed by quantitative autoradiography could have been used. To quantify the number of AChRs per myotube. Alternatively, sequential labeling of myotube cultures with cold α-BTX and ^{125}I-α-BTX (2-5 hours later) might have provided hints concerning the rates of AChR insertion at the membrane. This approach, which is essentially similar to the quantification of the total cell surface pool used in this study, may be more sensitive because it focuses the analysis on the rate of AChR insertion rather than on absolute numbers of AChRs. Finally, one could investigate only the post-infection insertion rate of AChR by incubating the myotube cultures with cold α-BTX just after infection, thereby effectively subtracting from the final results all the AChR that were present prior to mutant protein expression.

Effects of ras mutants and PD098059 on AChR clustering

First, a 40% reduction in the number of agrin-induced AChR clusters was observed when the dominant inhibitory ras mutant (N17) was expressed (figure 3.6). This reduction cannot be attributed to a drastic drop in AChR levels triggered by the expression of ras-N17 (figure 3.4), assuming that the levels of transgene expressed were similar in both experiments. It is important to note that a more thorough study, which would have included the small clusters, may have revealed a different scheme. Indeed, myotubes positive for ras-N17 frequently displayed constellations of punctate clusters that were clearly different from the ones observed in uninfected myotubes. Hence, it is possible that inhibition of ras function may block the formation and/or the consolidation of larger AChR clusters induced by agrin. One of the mechanism proposed for AChR clustering involves the gradual accretion of microaggregates into larger structures. The opposite mechanism, namely cluster dispersion involves the disassembly of the large aggregates into a panoply of small clusters (Olek et al., 1986; Krikorian and Daniels, 1989). Evidently, the fact that these observations may be based on partial inhibition of native ras (see previous section) complicates the analysis and might explain why the results sometimes appeared ambiguous. AChR cluster disassembly was observed in mice that have deficient TrkB signaling (Gonzalez et al., 1999). Since ras signals downstream of TrkB, it is possible that the true effect of ras-N17 is disassembly of pre-existing clusters.

A potentiation of the inductive effects of submaximal agrin was observed when MEK was inhibited by PD098059 (figure 3.7). This result appears to be in direct contradiction with a reduction of AChR clustering by ras-N17 since MEK activity should have been reduced. This apparent paradox signifies that a thorough study of the effects of ras-N17 on AChR clustering when expressed at levels demonstrated to be inhibitory is required. The drawback of this approach is that the levels of AChR would be expected to be decreased significantly, potentially hindering the clustering process. Incubating myotubes overnight with a concentration of PD089059 that significantly reduces the AChR pool would verify if such a reduction would prevent agrin-induced AChR clustering. These results raise important questions. Is MEK truly inhibitory for AChR clustering? Would MEK be part of the signaling pathway activated by TrkB, which negatively regulates clustering? Could ARIA signaling via MEK induce AChR expression but downregulate its movement on the membrane?

Second, in the absence of exogenous agrin, more AChR clusters were observed on myotubes expressing the mutant ras-V12-D38, but the inductive effects of maximal agrin were unaltered. This mutant caused an increase in AChR levels, which was not significantly different from the control. In contrast to ras-N17, the (low) expression level of ras-V12-D38 protein is expected to be less critical since in essence, it constitutively activates the downstream pathways. While ras-N17 is inhibitory because it sequesters ras-GEFs, making them unavailable for activation of native ras proteins (Feig and Cooper, 1988), dominant positive ras mutants constitutively activate the downstream pathway. Based on these findings, it appears as though activated ras partially mimics but does not potentiate the effects of maximal agrin. Hence, if ras is implicated in AChR clustering, it is probably part of the signaling cascade induced by MuSK because in general, activation of parallel signaling pathways potentiates the effects of agrin. For example, simultaneous addition of agrin and laminin results in a higher number of AChR clusters than when both reagents are added individually (Montanaro et al., 1998).

Ras-V12-D38 signals only via raf-1 because it cannot interact the other ras downstream signaling molecules with PI3K or ralGDS. Thus, unless ras-V12-D38 uses another downstream effector, it is assumed that raf-1 would mediate the clustering

process. In general, the raf-1 signaling cascade is thought to be almost entirely funnelled through MEK (Morrison, 1995). Therefore, at first, the results obtained with ras-V12-D38 appear to be in direct contradiction with that obtained with PD098059, which inhibits MEK (see above). A negative feedback loop between MEK and raf-1 has been reported in myoblasts because inhibition of MEK with PD098059 results in higher raf-1 activity (Coolican et al., 1997). Hence, if raf-1 signaling is implicated in AChR clustering, it would be via downstream effectors that are PD098059-insensitive. There is precedence in the literature for raf-1 signaling that does not implicate MEK (Laird et al., 1999), or in which the main substrate is not MEK but rather Tvl-1 (Lin et al., 1999) or Rb (retinoblastoma; Wang et al., 1998).

A recent report indicates that integrin α7 null mice display a striking constitutive activation of raf-1 in skeletal muscle (Saher and Hildt, 1999), and that this mutation results in a novel form of muscular dystrophy (Mayer et al., 1997). Based on the involvement of integrin α7 in both laminin β2- and agrin-induced AChR clustering in culture (Burkin et al., 1998), it came as a surprise that these mice had normal neuromuscular junctions (Mayer et al., 1997). The lack of phenotype was suggested to arise from compensation by other integrin forms. The interaction between integrins and the ras/raf-1/MAPK pathway is complex because the integrins can activate this pathway but a negative feedback loop via MAPK also inhibits integrin signaling (Hughes et al., 1997). Furthermore, it is possible that integrin α7 signaling downregulates raf-1 activity since its absence results in unregulated raf-1 activity. From these results, I conclude that raf-1 activity is certainly not deleterious for neuromuscular junction formation but I cannot say whether it plays a role in the process.

There has been a report, although unpublished, that rac could be involved in the signaling process downstream of MuSK (Weston C, 1998, Neurosci. Soc. Abstr.). Given that ras can activate rac (Nimnual et al., 1998; Qiu et al., 1995) via PI3K (Rodriguez-Viciana et al., 1997), it is plausible that the effects of ras on AChR clustering might be caused by a modulation of rac function. It is difficult to reconcile my results with this idea, as the ras mutant V12-D38 does not activate PI3K. Studies with ras-V12-Y40, which activates only PI3K signaling cascade, would have been informative.

Like ras-V12-D38, ras-V12 treated cells also displayed agrin-independent AChR clusters, an effect which is probably not the result of the 20% increase in surface AChR observed in these cells (figure 3.4). Indeed, to my knowledge, there is no report that an increase in AChR can, by itself, trigger AChR aggregation. In fact, tetrodotoxin (TTX), which blocks electrical activity in muscle, raises surface AChR levels by three fold but has not been reported to promote clustering (Bambrick and Gordon, 1994; Osterlund et al., 1989). However, the results obtained with ras-V12 were not always consistent and sometimes, the cells were unresponsive to agrin. In those occasions though, it was noticed that ras-V12 was cytotoxic for myotubes, causing extensive vacuolation. Since I did not investigate whether these cells were actually dying, I cannot draw conclusions on the exact consequences of expressing ras-V12 in myotubes.

The fact that ras-V12, but not ras-V12-D38, induces cell death is interesting in itself as it means the pathway leading to cell death does not utilize raf-1. This might not be surprising considering that raf-1 is often associated with cell survival (Majewski et al., 1999; Lau et al., 1998). In fact, it is possible that ras-V12 might signal via PI3K, which has been linked to ras-dependent apoptosis in skeletal muscle (Nakagawa et al., 1998). It is also possible that the difference in cytotoxicity between the two mutants is simply a reflection of different levels of protein expression. Evidently, one crucial improvement to all those experiments is to make sure that the protein levels of these mutants are not too low (ras-N17 and ras-V12-D38) nor too high (ras-V12), by carefully adjusting the MOIs or by inserting an inducible promoter in the adenovirus genetic background. Another approach to tackle this issue would be to make use of other ras-V12 point mutants like ras-V12-Y40, which activates only PI3K or ras-V12-E37, which activates only ralGDS (Rodriguez-Viciana et al., 1994). This approach would be useful because it would allow precise determination of which downstream effectors are used by ras during AChR clustering. For example, if ras-V12-D38 promotes AChR clustering but ras-V12-Y40 does not, one could conclude that ras probably uses raf-1 for this task, and not PI3K.

The effects of ras mutants on AChR clustering could also be due to non-specific modulation of other cellular processes. For example, the effects of the ras mutants on clustering might be due to interference with calcium signaling, which is known to be

important for AChR clustering (Rotzler et al., 1991;Megeath and Fallon, 1998;Grow et al., 1999). Indeed, the relationship between ras and calcium is complex since ras can modulate calcium signaling, and the inverse is true as well (Gawler, 1998). This issue could be investigated by incubating the ras-V12-D38 infected cells with a calcium chelator like BAPTA to see if the agrin-independent clusters would still form. It is also possible that both dominant active and inhibitory mutants might disrupt vital cellular processes such as transcription, translation, glycosylation, cytoskeletal rearrangements or even glucose transport (Dennis et al., 1989; Davis, 1993; Manchester et al., 1994).

The effects caused by H-ras mutants, especially the activated forms, might also reflect the activation of pathways not normally activated by agrin. Indeed, since cells express many different ras proteins with similar effector domains and downstream targets, it is possible that expressing activated H-ras might non-specifically contribute to the signaling pathway of R-ras or K-ras rather than H-ras. For example, forced expression of activated H-ras (ras-V12) in myoblasts effectively inhibited myogenesis (as expected), yet native H-ras was undetectable in myoblasts (this study). Continuing with this view, it is possible that effects observed with ras-V12 in myotubes might reflect non-specific activation of another homologous GTPase. The most likely candidate is RAP1 since it is aggregated at the neuromuscular junction (Pizon et al., 1996) and its effector domain and downstream targets are exactly similar to that of H-ras (Nancy et al., 1999). This unusual yet simple idea would be difficult to test experimentally and, if confirmed, would confound interpretations of results obtained with the ras mutants. Interestingly, RAP1 signals downstream of Trk (York et al., 1998), and mutation to the NPXY domain of TrkB abrogates its activation (Minichiello et al., 1998); this domain is conserved in MuSK (Valenzuela et al., 1995) and is crucial for AChR clustering (Zhou et al., 1999).

Collectively, the findings presented above suggest that ras might be playing a role in the process of AChR clustering. Since most of the data are preliminary and would require more experiments, it is clearly impossible to draw a firm conclusion on this issue. Unfortunately, due to time constraints, the most crucial experiments could not be performed (see future directions). Still, the groundwork to characterize and optimize this system should certainly facilitate future studies using recombinant adenoviruses. It was

quite unexpected that the ras mutants did not have more consequences on surface AChR proteins given that ras is involved in AChR transcription. While this fact facilitated the analysis of the AChR clustering experiments, it also raised the issue that the ras mutants were not as effective as expected. Alternatively, it might signify that changes in AChR mRNA observed with the ras mutants (Tansey et al., 1996) may not correlate directly with the protein levels at the membrane (this study).

Is ras downstream of MuSK?

The third objective was to determine if ras was downstream of MuSK. Given that expression of ras-N7 and ras-V12-D38 appeared to modulate AChR clustering, it is possible that ras might be implicated in MuSK signaling. In addition, the potentiation of agrin-induced aggregation by MEK inhibition also supports this assertion. These data are clearly insufficient to draw conclusions and these findings should be considered as preliminary. Nevertheless, they raise several important issues. First, if ras is involved in AChR clustering, it would add an unexpected level of complexity to the schemes already established since ras is already involved in induction of AChR transcription by ARIA/erbB (Tansey et al., 1996), and possibly in the negative regulation of agrin-induced clustering by neurotrophins/TrkB (Wells et al., 1999). Furthermore, if one considers the possible modulation of ras by integrins ($\alpha 7\beta 1$ and $\alpha v\beta 1$) or even dystroglycan (via Grb2?), then the picture becomes incredibly complicated (Burkin et al., 1998;Martin and Sanes, 1997;Yang et al., 1995). It would be an interesting challenge to determine how functional specificity would emerge from such a convergence of signals. Second, if agrin modulates ras function, it might explain why agrin has an effect on AChR transcription (Jones et al., 1996; Jones et al., 1997) and also why subsynaptic transcription occurs normally in rapsyn null mice (Gautam et al., 1999). Third, it would clarify why many factors, such as bFGF, HB-GAM, insulin or IGF-1, modulate AChR clustering even if they are not physiologically relevant (see table 2; Askanas et al., 1985; Peng et al., 1991; Dai and Peng, 1992; Peng et al., 1995; Baker and Peng, 1995). As an example of such convergence onto ras, consider the fact that both NGF and EGF modulate agrin transcription in PC12 cells, albeit at

different levels, but only NGF is thought to be physiologically relevant for neurons (Smith et al., 1997).

In conclusion, the system that has been used and optimized, i.e. the C2C12-CAR cells, appears to be suitable for studies using recombinant adenoviruses encoding ras or any other mutants. The results presented suggest that ras may play a role during AChR aggregation induced my agrin but also points out that much more work is required to draw firm conclusions. The idea that ras proteins are involved at synapses is not novel. Indeed, the subsynaptic expression of ras modulatory proteins such as synGAP (Kim et al., 1998; Chen et al., 1998) or CDC25Mm (Sturani et al., 1997) in neurons suggest that it might be implicated in synaptic plasticity. These preliminary data hint that ras may act by regulating synaptic formation *per se*.

Table 2. **Many factors that affect AChR transcription and/or clustering signal via ras.**

Factors	Receptor(s)	Ras signaling?	AChR transcription	AChR aggregation
ARIA	ErbB2/3/4	y	↑↑	-
Agrin	MuSK/MASC Integrins αvβ1 Dystroglycan	?	↑	↑↑↑
Neurotrophins	TrkB	Y	?	↓
Laminin	Integrins α7β1	y	?	↑
	Dystroglycan	? (Grb2)	?	↑
bFGF	FGFR	y	-	↑
HB-GAM	Syndecan RPTPz/β	Y	?	↑
CGRP	CGRPR	?	↑	-
IGF	IGFR	Y	-	↑

Future directions and experimental improvements

1) One of the most important experiments would be to investigate whether or not agrin/MuSK signaling can modulate ras activity. This could be achieved by comparing the relative amount of GTP versus GDP bound to ras before and after agrin treatment. Two approaches could be used to reveal the GTP/GDP loading of ras. First, after incubating the cells with radioactive nucleotides and ras immunoprecipitation, the nucleotides are eluted from the ras proteins and processed by thin-layer chromatography to separate the GDP from GTP (Satoh et al., 1990).

The alternative method is similar but does not require handling of radioisotopes. The levels of GDP and GTP bound to ras are indirectly but very accurately revealed using a luciferase assay (Scheele et al., 1995). Using a series of enzyme, which modify both GDP and/or GTP into ATP, one can monitor the relative amounts of GDP and GTP that were eluted from the immunoprecipitated ras proteins. I started to use this technique but I have not been able to test the GTP/GDP load of ras because optimization of the procedure for low amount of ATP (fM) was difficult.

2) Another important modification relates to the amount of agrin that was used for most of the clustering assays. It has been shown that addition of 100pM of c-agrin$_{4,8}$ to C2C12 myotubes induces the maximal amount of AChR clusters. Therefore, using submaximal agrin concentrations (below 100pM) would be more informative, especially to determine whether activated ras mutants can potentiate the effects of c-agrin$_{4,8}$.

3) A thorough study that would include both small and large AChR clusters would be required to draw conclusions on the nature of the effects of the ras mutants given the ambiguities generated by this study. It is thought that AChR clustering occurs in two waves with the formation of microaggregates preceeding their coalescence into larger structures (Anderson MJ and Cohen MW, 1977; Steinbach, 1981; Montanaro et al., 1998). Hence, it would be instructive to compare the clustering process in non-infected myotubes at earlier time points with that of ras-N17-expressing myotubes, for example.

4) Ideally, these experiments should be performed with many different activated ras mutants bearing point mutations in their effector domain, in order to pinpoint which specific signaling pathways are involved. The results with ras-V12-D38 indicate that ras signaling during the clustering process probably occurs via raf-1. This statement would be reinforced if, for example, ras-V12 mutants that cannot activate raf-1 were shown to have no effect on clustering.

5) In order to investigate further the possible involvement of raf-1 in the clustering process, one could test whether it is activated by agrin. This could be determined by assaying whether it is translocated to the membrane after it becomes phosphorylated on tyrosine residues (Leevers et al., 1994; Xia et al., 1999). Alternatively, the introduction of kinase dead or activated raf-1 (raf-1-CAAX) mutants into myotubes would also address this question nicely (Stokoe et al., 1994).

6) It might be good to consider using an inducible promoter instead of the CMV promoter used in this study. This would permit the infection of myoblasts without preventing myogenesis and would allow the experimenter to be able to control the activity of the promoter at will (Rossi and Blau, 1998). As a consequence, the protein levels would be similarly regulated since the activity of the promoter is proportional to the amount of inducer added to the cells. Furthermore, one could also test whether myotubes free of mutant proteins at the time of agrin treatment respond normally (for an excellent example, see Gossett et al., 1988). It is evident that this approach would be particularly useful with ras-V12 since it would be possibleto optimize the duration of the expression as well as the protein levels so as to minimize its cytotoxicity.

Chapter 4

Shc does not signal downstream of MuSK

Introduction

Shc is an adaptor protein that is implicated in a variety of signaling cascades as it couples activated receptor tyrosine kinases (RTK) to their downstream effectors. Typically, Shc binds to RTK via its phosphotyrosine binding (PTB) domain, which recognizes specific phosphotyrosine-containing motifs (Borg and Margolis, 1998). It is known that Shc PTB domains has a preference for a motif containing NPXY in the juxtamembrane domain of many RTK (Kavanaugh et al., 1995; Isakoff et al., 1996). This interaction results in Shc tyrosine phosphorylation, thereby creating docking sites for other signaling proteins, such as Grb2. It is noteworthy that tyrosine phosphorylation is observed on only two of the three Shc isoforms: on p47 and p56 but not on p66.

A role for Shc during neuromuscular junction formation (NMJ) is primarily suggested by the fact that MuSK and Trk RTK, which share highly homologous intracellular tails, both possess a juxtamembrane NPXY motif (see figure 4.1) that has been shown to be important for their respective functions (Zhou et al., 1999; Minichiello et al., 1998). Since Shc strongly interacts with Trk receptors upon activation by neurotrophins (Dikic et al., 1995), it is possible that a similar interaction occurs between MuSK and Shc. Furthermore, downregulation of agrin inductive effects by neurotrophins/TrkB signaling suggests that both Trk and MuSK might be competing for a limited pool of common intracellular target, such as Shc.

Based on these findings I decided to investigate if Shc is a downstream effector of MuSK. The results suggest that Shc probably does not play a role downstream of MuSK since neither Shc tyrosine phosphorylation nor its interaction with MuSK was modulated consistently by agrin.

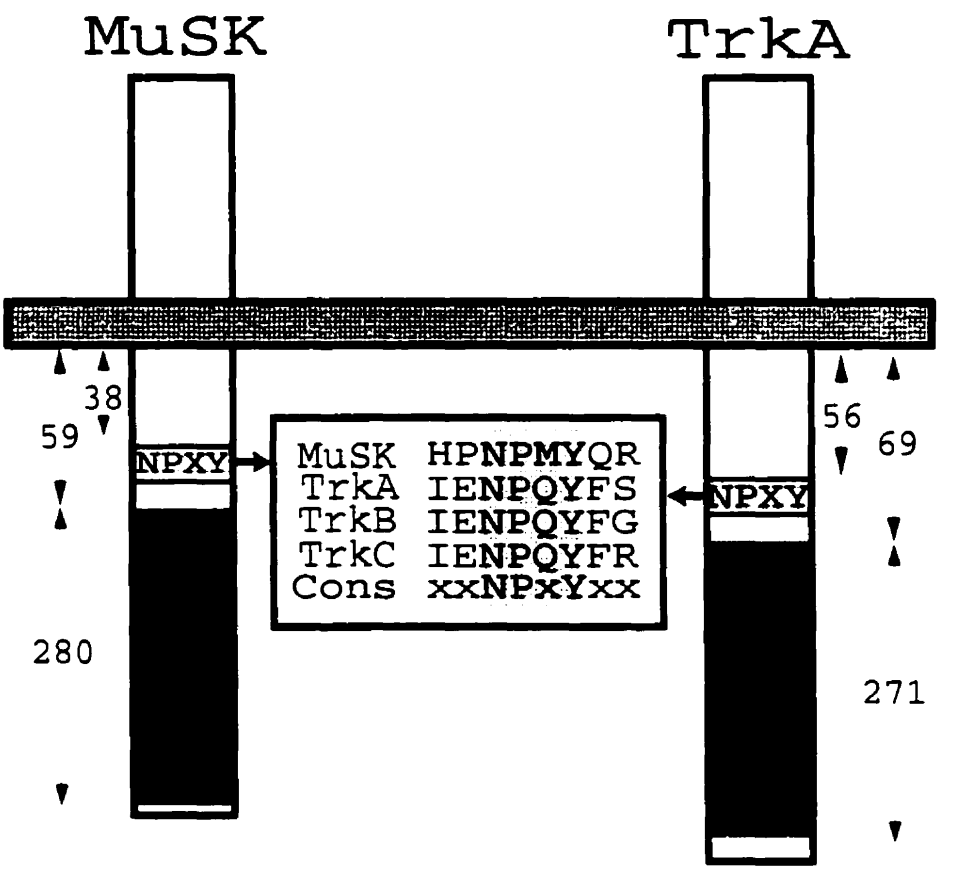

MuSK
TrkA
38
59
NPXY
280
MuSK HPNPMYQR
TrkA IENPQYFS
TrkB IENPQYFG
TrkC IENPQYFR
Cons xxNPxYxx
56
69
NPXY
271

Figure 4.1 **The NPXY domains of MuSK and Trks**. The intracellular tails of MuSK and Trk receptor tyrosine kinases are highly homologous, especially in the kinase domain (shaded area). The only other region of functional importance (at least in the context of Trk) that is conserved is the NPXY motif located in the juxtamembrane region. The tyrosine within this motif is phosphorylated in both RTK and is crucial for their function. In the case of Trk, it involves the binding of the protein Shc via a PTB domain. Another major phosphotyrosine of Trk is located after the kinase domain and is involved in the activation of PLC-γ; this tyrosine is absent from MuSK. The NPXY domains are situated at similar distance from the membrane in both RTKs. In the rectanlge, the sequences of the NPXY motifs of MuSK, TrkA, TrkB and TrkC are aligned (Cons is the consensus motif).

Results

Shc expression and tyrosine phosphorylation

I first confirmed that the three Shc forms (47, 56 and 66kD) are expressed in C2C12 cells (figure 4.2 A). Probing an extract of A431 cells known to contain high levels of Shc (figure 4.2A) insured the specificity of the antibodies used. Subsequently, I investigated whether Shc tyrosine phosphorylation was modulated by exposure to agrin concentration known to induce maximal MuSK activity (1nM). It was found that the tyrosine phosphorylation levels of both p47 and p56Shc were not altered significantly by agrin treatment (figure 4.2 B). It is noteworthy that the basal levels of Shc tyrosine phosphorylation were quite high, potentially masking the agrin-dependent changes. I found that growing the cells in serum-deprived medium had no effect on the extent of Shc tyrosine phosphorylation (figure 4.2 C).

In C2C12 cells, the endogenous phosphorylation levels of p47 and p56 forms of Shc were much higher than that observed for p66. No induction of tyrosine phosphorylation was apparent after agrin treatment (1nM, 30 minutes). It is well accepted that the different anti-phosphotyrosine antibodies available do not necessarily recognize all phosphotyrosines with the same efficiency. For example, the monoclonal antibody 4G10 recognizes activated MuSK very well but PY20 does not (unpublished observations). I therefore tried another phosphotyrosine antiserum, more precisely RC20H that is a Fab' fragment conjugated to HRP that is derived from PY20. Interestingly, I saw a difference in the pattern of Shc phosphorylation when using this antibody (figure 4.3 A). It is notable that p56Shc was more phosphorylated than p47; phosphorylation levels of p66Shc, if present, were always much lower than that of p47 and p56Shc. Densitometric analysis showed that the increase in p56Shc phosphorylation in response to agrin was modest when the values were normalized to the actual levels of Shc proteins present (figure 4.3 B). Similarly, the phosphorylation differences for p47Shc, although perceptible, were modest. Despite this, it appeared as though Shc tyrosine phosphorylation pattern is modulated by agrin, at least in the context of this experiment. Moreover, the peak at 30 minutes correlates well with the temporal activation of MuSK after agrin treatment. It is

noteworthy that even with RC20H, I could not consistently see an increase in Shc tyrosine phosphorylation in response to agrin. Hence, I conclude that the increase in Shc tyrosine phosphorylation that is observed in figure 4.3B does not reflect accurately the situation.

Shc subcellular localization

I was then interested to see if the subcellular localization of Shc was affected by agrin since Shc is known to translocate from an internal pool, associated with the cytosol and the cytoskeleton, to the membrane after RTK activation. In order to this, I took advantage of the fact that extraction with Triton X-100 solubilizes membrane-associated proteins whereas RIPA, which is a much stronger solubilizing agent (it contains Triton X-100 along with SDS and sodium deoxycholate), extracts proteins bound to the cytoskeleton (Schlaepfer et al., 1998). Extractions of myotubes (treated with c-agrin as indicated) from identical cultures were done with either Triton X-100 or RIPA (RIPA1) and the pellet from the Triton X-100 extraction was further solubilized using RIPA (RIPA2). As shown in figure 4.4 A, there is an increase in tyrosine phosphorylation in a band of approximately the size of p56Shc in the Triton X-100, but not in the RIPA2, indicating that phosphorylated Shc is anchored to the membrane. It is noteworthy that the blot was stripped and reprobed with an anti-Shc serum to insure that the phosphorylated band was indeed p56Shc. The increase in tyrosine phosphorylation after agrin treatment was relatively modest after normalization for protein content in each lane (figure 4.4 B). Interestingly, the level of Shc tyrosine phosphorylation observed in RIPA1 fractions was 70% higher after agrin exposure indicating that a portion of the modified Shc might already have translocated to the cytosol. Of note, only half of the Shc pool was extracted by Triton X-100, the other half being solubilized by RIPA (RIPA2). This finding was supported by the fact that Shc levels in RIPA1 were definitely higher than both Triton and RIPA2 individually. Interestingly, agrin treatment did not induce any changes in the levels of Shc associated with the membrane.

Do Shc and MuSK interact?

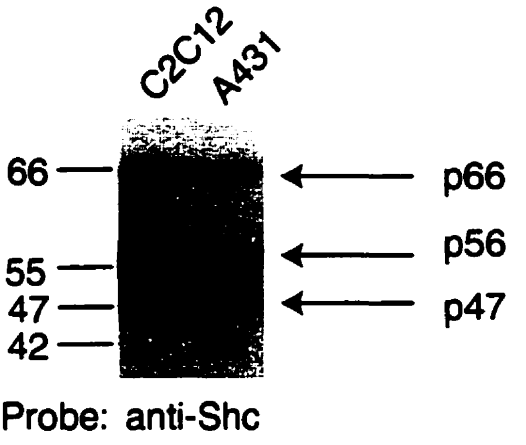
Next, I tested whether activated MuSK could interact with Shc since MuSK has a NPXY motif that is crucial for its function (Zhou et al., 1999). First, I attempted to co-immunoprecipitate MuSK with Shc. On many occasions, I could co-immunoprecipitate a phosphoprotein of 110kD that co-migrated with MuSK and the time course of association correlated with MuSK activation (figure 4.5 A). Although unlikely, it was possible that another RTK of similar molecular weight of MuSK and activated by agrin was co-immunoprecipitated with Shc. To resolve this question, I probed a Shc immunoprecipitation with an anti-MuSK antiserum: I found that it recognized a band at 110kD (figure 4.5 B). Even though the association of Shc with MuSK appears to be slightly induced by agrin, the phosphorylation level of Shc (RC20H) was unchanged throughout the time course, a finding that is inconsistent with the usual function of Shc. Indeed, when Shc is associated with RTK, it usually becomes increasingly phosphorylated. It is noteworthy that probing twin blots with the secondary antibodies alone did not yield any signal indicating that the signals observed were likely to be specific. Importantly, an identical immunoprecipitation from Triton extract of cultured myotubes with an anti-MuSK antiserum insured that the activation of MuSK by agrin was indeed observed. Finally, agrin treatment did not modulate the binding of Grb2 to Shc in any way indicating that if MuSK signals via Shc, it does not involved Grb2.

It was possible that the antibody used for immunoprecipitating Shc caused the variability of the results. For example, it may not recognize the epitope as well when Shc was bound to MuSK. Therefore, the reverse co-immunoprecipitation was attempted, that is immunoprecipitate MuSK and probe for Shc. I suspected that the success rate of this procedure should be higher than the contrary because the pool of MuSK is relatively small and can be depleted from the cell extract quite easily; in contrast, the levels of Shc proteins are much higher and it proved difficult to deplete it completely (data not shown). My efforts were impeded because Shc proteins run at the same molecular weight as the heavy chain of the antiserum used for immunoprecipitating MuSK (figure 4.6 A; see Material and Methods). A 10 fold dilution of the serum revealed a protein distinct from the IgG just above 55kD that was recognized by an anti-Shc antibody and appears to be induced by agrin. Still, the presence of the intense signal from the heavy chain was not optimal.

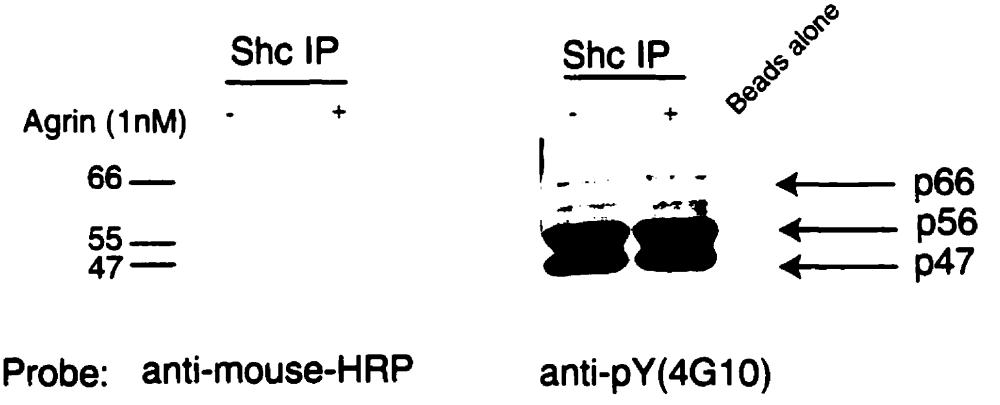
Cross-linking the anti-MuSK antiserum to the protein-G agarose beads (see Material and Methods) completely removed the IgG band without interfering with the immunoprecipitation efficacy (figure 4.6 B). Furthermore, I was able to visualize clearly that both p47Shc and p56Shc were associated with activated MuSK. Unfortunately, since the zero time point was not cross-linked, I do not know if this interaction was induced by agrin. This procedure was attempted several other times but I was never able to get signals as clear as these (see figure 4.7). The fact that the secondary antibody alone did not reveal intense signals indicates that Shc was indeed specifically recognized in the MuSK immunoprecipitation.

Figure 4.7 shows a typical experiment where I tried to investigate all variables simultaneously. In this case, Shc association with MuSK was very strong but was not induced by agrin at all (first set of blots to the left, top part). In contrast, the levels of Shc proteins in the MuSK immunoprecipitations increased with agrin treatment, although a substantial amount was present even in the absence of agrin (first set of blots to the left, bottom). The phosphorylation level of MuSK was increased in both Shc and MuSK immunoprecipitations, but that of Shc was only increased in the MuSK immunoprecipitation (it was unchanged in the Shc immunoprecipitation; second sets of blots, to the right). The differences observed were likely to be quantitative since the protein levels were similar in the Shc and MuSK immunoprecipitations (probed for Shc and MuSK, respectively), as well as in the b-tubulin loading control. Note the stronger signal in both immunoprecipitations of a 60kD phosphoprotein in agrin-treated lanes; the identity of this protein is unknown.

A


C2C12
A431
66
55
47
42
p66
p56
p47
Probe: anti-Shc

B


Shc IP
Agrin (1nM)
- +
66
55
47
Shc IP
Beads alone
- +
p66
p56
p47
Probe: anti-mouse-HRP
anti-pY(4G10)

C

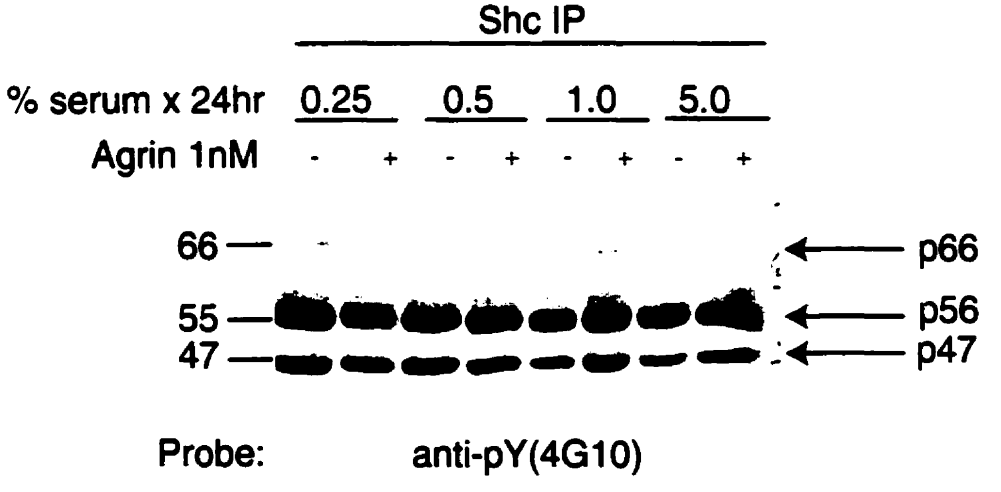
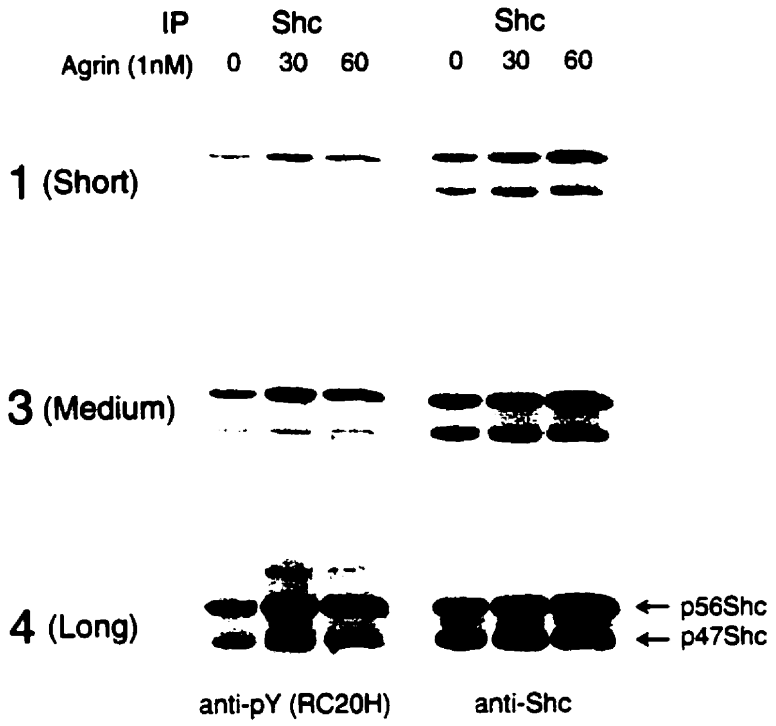

Shc IP
% serum x 24hr
0.25
0.5
1.0
5.0
Agrin 1nM
- + - + - + - +
66
55
47
p66
p56
p47
Probe:
anti-pY(4G10)

Figure 4.2 A **Shc is expressed in C2C12 cells.** Shc was immunoprecipitated from a Triton-based cell lysate of C2C12 myotubes with a polyclonal anti-Shc serum. After processing with SDS-PAGE (10%) and transfer on nitrocellulose membrane, immunoblotting was performed with a monoclonal anti-Shc antiserum. Three co-migrating Shc isoforms are similarly expressed in C2C12 myotubes and A431 cells, the positive control.

Figure 4.2 B **Shc basal tyrosine phosphorylation**. Cultured myotubes were extracted with Triton buffer and immunoprecipitated with an anti-Shc antiserum. Following SDS-PAGE and electrotransfer, the levels of Shc tyrosine phosphorylation were assessed with monoclonal antibody 4G10. The efficiency of the stock of recombinant agrin used for these experiments was insured by looking at MuSK activation (data not shown; see figure 4.5 A). The signal was not due to non-specific binding to the protein-G agarose beads used for immunoprecipitating the antibody-protein complex (see beads alone lane). Furthermore, the specificity of the signal recognized by 4G10 was demonstrated by omitting the primary antibody (4G10) on parallel lanes (see anti-mouse-HRP panel).

Figure 4.2 C **Shc basal tyrosine phosphorylation is unaffected by serum starvation.** Differentiated C2C12 cultures were incubated overnight with fusion medium containing different amounts of horse serum (the usual amount is 4%). The following day, the cells were extracted and processed exactly as described in figure 4.2 B.

A


IP
Shc
Shc
Agrin (1nM)
0
30
60
0
30
60
1 (Short)
3 (Medium)
4 (Long)
p56Shc
p47Shc
anti-pY (RC20H)
anti-Shc

B

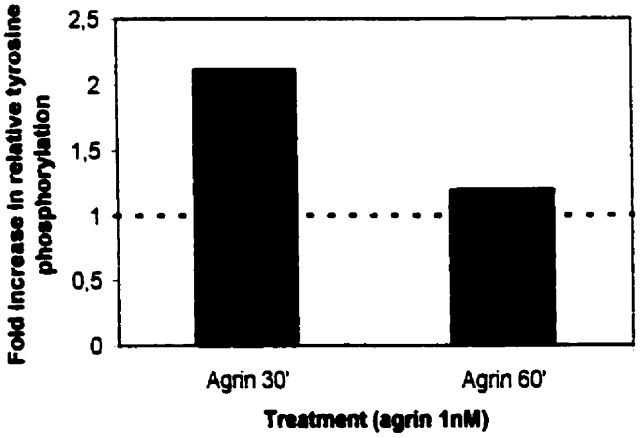
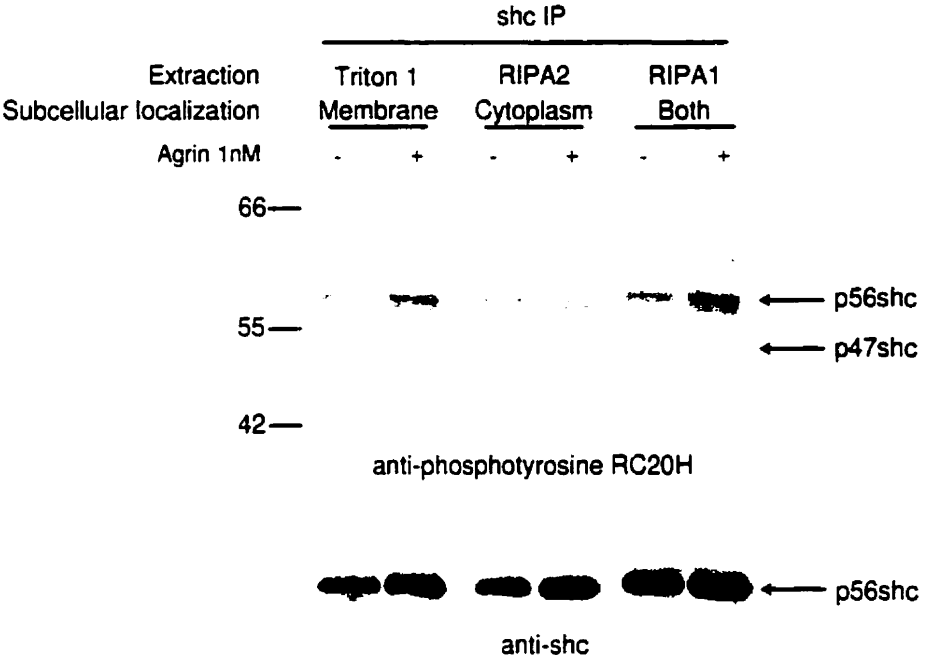

Fold increase in relative tyrosine phosphorylation
2,5
2
1,5
1
0,5
0
Agrin 30'
Agrin 60'
Treatment (agrin 1nM)

Figure 4.3 **RC20H recognizes a different pattern of Shc tyrosine phosphorylation.** C2C12 myotubes were exposed to agrin (1nM) for 30 minutes, immunoprecipitated with an anti-Shc serum and processed with SDS-PAGE followed by transfer on nitrocellulose membrane. Identical blots were generated. The first one was probed with RC20H, an anti-phosphotyrosine Fab' fragment conjugated to HRP. The second was probed for Shc to normalize the phosphotyrosine content to the actual levels of Shc proteins immunoprecipitated. RC20H and 4G10 appear to recognize different phosphotyrosine motifs since RC20H reveals lower amount of basal tyrosine phosphorylation. The result of a densitometric analysis are presented at the bottom. Four different exposure time of the blot were scanned to account for differences in signal intensities. The relative intensity of the phosphorylated bands was assessed and normalized with the values obtained for Shc protein levels of similar exposure time.

A


shc IP
Extraction
Subcellular localization
Triton 1
Membrane
RIPA2
Cytoplasm
RIPA1
Both
Agrin 1nM
- + - + - +
66
55
42
p56shc
p47shc
anti-phosphotyrosine RC20H
p56shc
anti-shc

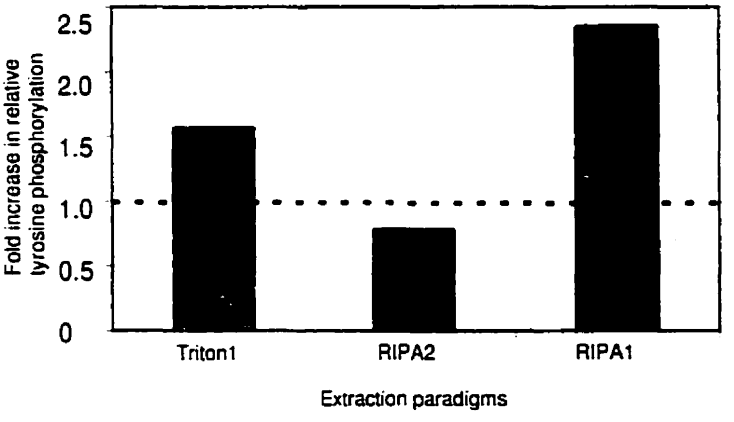
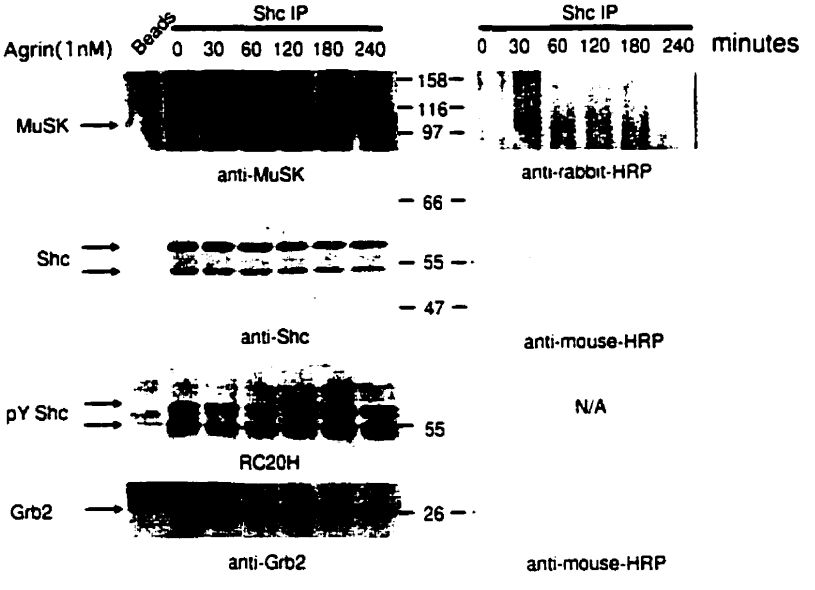

Fold increase in relative tyrosine phosphorylation
2.5
2.0
1.5
1.0
0.5
0
Triton1
RIPA2
RIPA1
Extraction paradigms

Figure 4.4 **Phosphorylated Shc is associated with the membrane fraction.** Shc interaction with activated receptor tyrosine kinases generally results in Shc tyrosine phosphorylation. In order to see if Shc phosphorylated in response to agrin was located at the membrane, differential extractions were performed using two extraction buffers. Triton X-100 based buffers is expected to solubilize mainly membrane-associated proteins whereas RIPA buffer is able to solubilize the cytoskeleton-associated membrane protein as well. One dish of myotubes was extracted with RIPA alone (RIPA1). The other one was extracted first with Triton (Triton1), the Triton insoluble material (pellet) was further solubilized in RIPA buffer (RIPA2). Shc immunoprecipitations were performed on each cell lysate and analyzed by western blotting. Top, Identical blots were probed with RC20H or Shc antiserum. The phosphorylated band co-migrated with that of p56Shc. Bottom, The graph illustrates the fold increase in tyrosine phosphorylation of p56Shc after normalization to protein content densitometric analysis. Quantification was done as described in the legend of figure 4.3 except that only one exposure time was used.

A


MuSK IP
Shc IP
Agrin(1nM)
0 5 10 30 60
0 5 10 30 60 minutes
116
pY MuSK
97
anti-pY (4G10)
anti-pY (4G10)
Exposure:
20 seconds
60 seconds

B


Shc IP
Shc IP
Agrin(1nM)
Beads
0 30 60 120 180 240
0 30 60 120 180 240 minutes
158
116
97
MuSK
anti-MuSK
anti-rabbit-HRP
66
55
47
Shc
anti-Shc
anti-mouse-HRP
pY Shc
55
N/A
RC20H
Grb2
26
anti-Grb2
anti-mouse-HRP

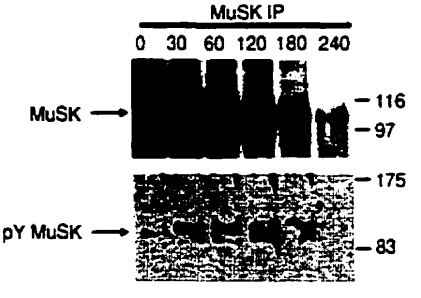
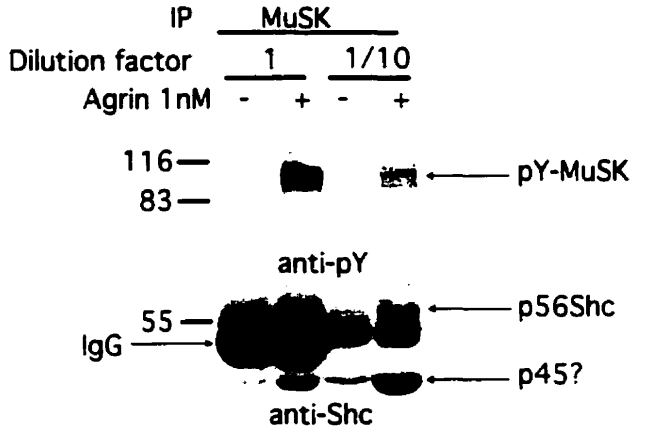

MuSK IP
0 30 60 120 180 240
MuSK
116
97
175
pY MuSK
83

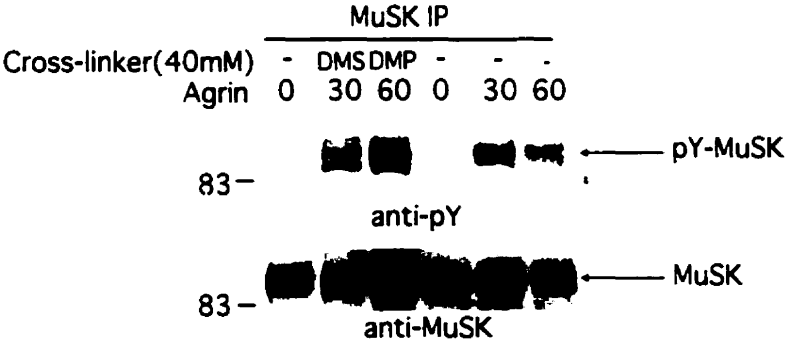
Figure 4.5 A **110 kD phosphoprotein is co-immunoprecipitated with Shc**. Myotubes treated with or without agrin (1nM) for different lengths of time (0, 5, 10, 30 and 60 minutes) were extracted with a Triton buffer. The extracts were separated in two equal volumes and Shc and MuSK immunoprecipitations were performed independently. Equal volumes were loaded in a 10% SDS-PAGE and transfered on nitrocellulose filters as described in Material and Methods. Western blot analysis was done with phosphotysoine antibody 4G10.

Figure 4.5 B **MuSK is bound to Shc and agrin does not modulate Shc/Grb2 interaction.** C2C12 myotubes were exposed to agrin (1nM) for various lengths of time (0, 30, 60, 120, 180 or 240 minutes) and processed exactly as described in legend of figure 2.5 A. The nitrocellulose membrane was first probed with anti-MuSK, anti-Shc and anti-Grb2 antisera; the levels of Shc phosphorylation was determined by backprobing the stripped filter with RC20H.

A


IP
MuSK
Dilution factor
1
1/10
Agrin 1nM
- + - +
116
83
pY-MuSK
anti-pY
55
IgG
p56Shc
p45?
anti-Shc

B


MuSK IP
Cross-linker(40mM)
- DMS DMP - - -
Agrin
0 30 60 0 30 60
83
pY-MuSK
anti-pY
83
MuSK
anti-MuSK

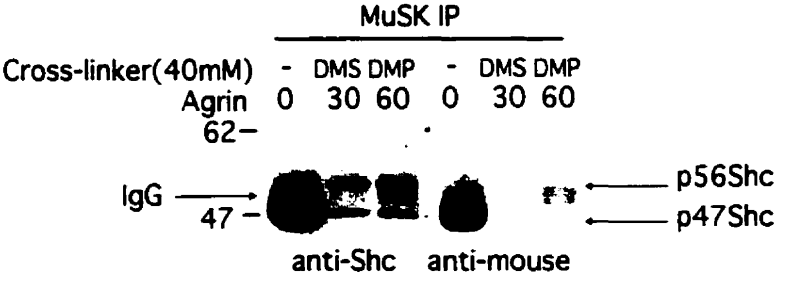

MuSK IP
Cross-linker(40mM)
- DMS DMP - DMS DMP
Agrin
0 30 60 0 30 60
62
IgG
47
p56Shc
p47Shc
anti-Shc
anti-mouse

Figure 4.6 A **Shc is associated with MuSK**. MuSK immunoprecipitation was performed on Triton extracts from myotubes exposed to agrin (1nM ; 30 minutes). Using undiluted serum, a high amount of MuSK was recovered as assessed by probing with 4G10. Analysis of Shc co-immunoprecipitation was hindered by the large IgG band that was strongly reactive with both the primary and secondary antibodies. A dilution by a factor of 10 reduced considerably the intensity of the IgG band and a protein of 56kD was recognized in the agrin-treated lane only.

Figure 4.6 B **Cross-linking anti-MuSK antiserum to beads does not affect immunoprecipitation efficiency and facilitates Shc probing.** In an attempt to solve the analytical problem generated by the presence of the IgG band in MuSK immunoprecipitations, I cross-linked the antibodies to protein-G agarose beads (see Material and Methods). Two different cross-linkers were used (DMS and DMP) but no differences were noticed between the two. This procedure did not affect the efficiency of MuSK immunoprecipitations (compare the first and second lanes) and removed the IgG bands (see anti-mouse blot).

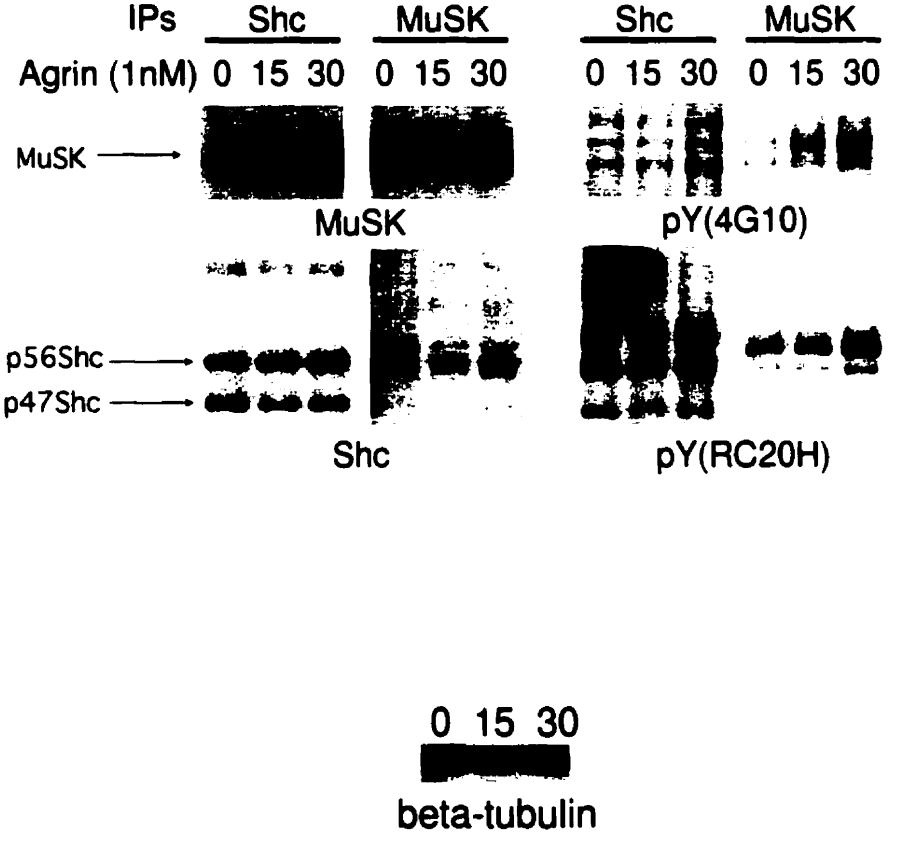

IPs
Shc
MuSK
Shc
MuSK
Agrin (1nM) 0 15 30 0 15 30 0 15 30 0 15 30
MuSK
MuSK
pY(4G10)
p56Shc
p47Shc
Shc
pY(RC20H)
0 15 30
beta-tubulin

Figure 4.7 **The effects of agrin on the interaction between Shc and MuSK are ambiguous.** Myotubes exposed to agrin were extracted with Triton buffer and the resulting cell lysates were separated in two for immunoprecipitation with Shc and MuSK antibodies. In this experiment, MuSK antiserum was cross-linked as described in Material and Methods (see also figure 4 .6 B). Western blot analysis with antibodies against Shc, MuSK or phosphotyrosine (4G10 and RC20H, top and bottom pannels, respectively). MuSK has been observed to run as a doublet under certain conditions.

Discussion

The signal transduction downstream of MuSK, a receptor tyrosine kinase involved in the neuromuscular junction formation, is at present unknown. In contrast, the signaling pathways used by Trk receptors are very well worked out (Kaplan, 1998). Since MuSK and Trk have highly homologous kinase domains (Valenzuela et al., 1995), it might be instructive to investigate whether known Trk targets such as PLCγ, Shc or PI3K are involved in MuSK signaling. Outside of the kinase domains, the only conserved portion is a NPXY motif located in their juxtamembrane domain. In the context of activated Trk, this motif acts as a docking site for the adaptor protein Shc (Kavanaugh et al., 1995) and is important for its signaling (Yoon et al., 1997; Minichiello et al., 1998; Hallberg et al., 1998). Based on these data, I decided to test whether Shc is an effector for activated MuSK.

It is well established that when Shc interacts with activated receptor tyrosine kinases, it becomes rapidly phosphorylated on tyrosine residues located in the collagen-homology domain (Pelicci et al., 1992). Hence, my first goal was to test whether Shc phosphorylation was induced by agrin treatment. Initial attempts showed that agrin treatment did not influence the levels of Shc phosphorylation, but it also highlighted the fact that the increase might have been hindered by high basal tyrosine phosphorylation of Shc (figure 4.2B). Several groups have shown that the basal phosphotyrosine content of Shc is often high in skeletal muscle, suggesting that this phenomenon is not uncommon (Chow et al., 1996; Paez-Espinosa et al , 1998). Furthermore, the increase in tyrosine phosphorylation after ligand treatment has been shown to be relatively modest in the same cells (Altiok et al., 1997; Tsakiridis et al., 1998). High basal activity of normal cellular processes is a common problem in signaling studies and incubating the cells in serum-free medium commonly circumvents it. This modification had no effect on Shc phosphotyrosine content in C2C12 myotubes (figure 4.2 C), indicating that the source of Shc phosphorylation probably originated from signaling induced by autocrine factors. For example, it could be caused by neurotrophin- or muscle ARIA-induced signaling, as these factors are constitutively secreted by myotubes and their receptors are expressed on the cell surface (Wells et al., 1999;Zhu et al., 1995).

In one study, the increase in phosphotyrosine content of Shc in response to insulin-like growth factor-1 was very significant, despite basal tyrosine phosphorylation (Ewton et al., 1998). The only major difference between their protocol and the others (mentioned above) was that they used a different phosphotyrosine antibody. I had observed that distinct patterns of phosphotyrosine patterns emerged when a MuSK immunoprecipitation was analyzed by immunoblotting with different phosphotyrosine antibodies and a strong signal was found with 4G10, but not with PY20 (data not shown). Although these antibodies were not generated against phosphopeptides (Ohtsuka et al., 1984), it appears that intrinsic differences in affinity for specific phosphorylated sites may exist. When I probed a Shc immunoprecipitation with RC20H (a PY20 variant) instead of 4G10, it appeared as though the level of basal tyrosine phosphorylation of Shc was lower, uncovering a modest increase in phosphorylation in response to agrin (figure 4.2 and 4.3). Still, I was unable to observe a consistent agrin-dependent increase in Shc phosphorylation. From these results, I concluded that Shc tyrosine phosphorylation is not modulated by agrin. Additional support for this assertion came from the observation that the interaction between Grb2 and Shc, which is dependent on Shc tyrosine phosphorylation, was unaffected by agrin (figure 4.4B). This also indirectly indicated that ras activity is probably high in C2C12 cells since Shc phosphorylation and association with Grb2 generally correlates well with the degree of activation of the ras/MAPK pathway (Lin and Abraham, 1997). Importantly, this finding stipulates that if MuSK activates ras during neuromuscular junction formation, as hypothesized in Chapter 2, it is probably not via Shc and Grb2.

In a parallel set of studies, I tried to determine if Shc could bind to MuSK and, importantly, if the interaction was regulated by agrin. Indeed, a signaling protein that has a physiologic role downstream of MuSK would be expected to respond to agrin. An interaction between MuSK and Shc was observed many times, and the controls performed with secondary antibody alone and/or with beads alone indicate that it was probably not due to experimental artifacts, like non-specific cross-reactivity to the antibodies (figure 4.5 B; figure 4.6). On some occasions, the interaction appeared to be clearly modulated by agrin (figure 4.5 A), but again, I failed at repeating those findings consistently. Indeed,

sometimes no interaction was visible between the two proteins whereas other times, it was constitutive (i.e., not induced by agrin). In order to clarify this issue, I conducted experiments during which all the aspects of the interaction were investigated simultaneously. As shown in figure 4.7, although some aspects of the interaction appeared to be modulated by agrin, but others were not. For instance, the interaction between MuSK and Shc was constitutive and Shc phosphorylation was not induced. So far, such a constitutive interaction between Shc and RTKs has only been reported in cells expressing activated (oncogenic) RTKs such as erbB2 (Stevenson and Frackelton, Jr., 1998) or Trk (Borrello et al., 1994; Pelicci et al., 1995; Watson et al., 1999). Since MuSK activity in the absence of agrin is very low, this constitutive interaction could also be caused by a phosphotyrosine-independent interaction. Even though such an interaction between Shc and the protein phosphatase PTP-PEST has been reported (Charest et al., 1996), it is incompatible with the idea that phosphorylation of the tyrosine contained in the NPXY motif is crucial for MuSK activity (Zhou et al., 1999).

This inconsistent behavior suggests that the interaction I observed might also be due to a non-specific binding of the two proteins. It is generally assumed that the interaction between two proteins is specific if the complex is intact once the co-immunoprecipitation has been subjected to washes in a buffer containing a high concentration of NaCl (500mM instead of 150mM). When I performed such a treatment, the interaction was not observed (data not shown). This approach, which is valuable mainly for protein-protein interactions that are relatively strong, might not have been optimal for studying the interaction between Shc and MuSK. Indeed, the strength of their association is predicted to be relatively weak since MuSK does not contain some crucial amino acids located upstream of NPXY domain; more precisely, a hydrophobic residue at –5 and an asparagine at position –3 from the tyrosine (Zhou et al., 1995). These residues are important because if they are present, the interaction is considerably stronger (e.g. Trk; Dikic et al., 1995) than when they are absent (insulin receptor; van der et al., 1999; Laminet et al., 1996). Still, while it is very difficult to co-immunoprecipitate the insulin receptor with Shc, the increase in Shc phosphorylation observed indicated that they somehow interacted (VanderKuur et al., 1995).

A recent report by Zhou et al. (1999) answered a lot of the questions generated by the ambiguous results I obtained. Indeed, introduction of a full length MuSK into MuSK -/- cells restored agrin-induced AChR clustering, only when the ATP binding site in the kinase domain or NPXY motif were unaltered. When the tyrosine of the NPXY motif was mutated to a phenylalanine, the effects of agrin were abolished and spontaneous clustering was also absent. It is known that such a motif, in the context of activated receptor tyrosine kinases, is a favorable binding site for protein bearing PTB domains, like Shc or PLCγ (Kavanaugh and Williams, 1994; Borg and Margolis, 1998). Hence, it is likely that this important MuSK downstream effector is a PTB domain-containing protein. Confirming my results, they did not see a significant increase in Shc tyrosine phosphorylation in response to agrin in cells expressing wild type MuSK. Since the strength of the interaction between Shc and MuSK is predicted to be weak (see above), an optimal Shc binding site that resembled that of Trk receptors was introduced upstream of MuSK's NPXY motif by replacing the sequence DRLHP for IPILE. A similar approach dramatically increased the affinity of the insulin receptor for Shc (van der et al., 1999). For MuSK, this modification did not increase the ability of MuSK to promote clustering but actually led to a reduction in the effects of agrin. Based on these results, they concluded that Shc was not required for agrin signaling. Similar results were obtained when the same approach was use for another candidate, namely PLCγ. The results presented in this article were impressive and led the authors to suggest that a Shc-like protein, but not Shc itself, with a PTB domain, is crucial for MuSK activity.

Taken collectively, these results strongly support the notion that Shc is not important in the signaling cascade activated by agrin. Consequently, I can conclude that the interaction that I observed between Shc and MuSK was probably non-specific and due to a chance occurrence. An alternative explanation is that a low level of MuSK activity is present in myotubes, either caused by intrinsic MuSK activity or by an unknown endogenous MuSK ligand. This MuSK activity, which could be involved in spontaneous AChR clustering, might also promote the binding of Shc to MuSK.

Future directions and experimental improvements

1) The fact that I used a single technique (co-immunoprecipitation) to investigate the interaction between Shc and MuSK was a limiting factor. This approach, which is valuable mainly for protein-protein interactions that are relatively strong, may not be optimal for studying the interaction between Shc and MuSK. To make sure that my results were valid, I should have used another technique. To do so, I could have used the method called a far western, which is similar to a typical western blot except that the filter is probed with a GST-fusion proteins instead of a primary antibody. In this instance, a MuSK immunoprecipitation generated from cells exposed to agrin could be probed with a Shc GST-fusion protein (Yeh et al., 1996). If the binding site is preserved after electrophoresis and electroblotting, it is possible to determine if the proteins interact together. The additional advantage of this approach is that I could also determine which domain of Shc is required for MuSK binding by using GST-fusion proteins of the different domains of Shc (SH2 and PTB).

2) The next step is obviously to identify the Shc-like protein that is important for MuSK signaling. As mentioned above, the NPXY domain of MuSK is crucial for its signaling but it does not signal via Shc but rather via another protein which possess a PTB domain (hence the protein is referred to as a Shc-like protein). This could be achieved two ways. First, a yeast-two hybrid screen using the juxtamembrane portion of phosphorylated MuSK as bait should permit the identification of proteins that interact specifically with this domain, even if the interaction is weak. Another option would be to screen a cDNA library from skeletal muscle with a series of probes aimed at identifying PTB domain-containing proteins since it is possible that this protein, like MuSK, is expressed exclusively in this tissue. In fact, it might be a good idea to perform such a study first on a the same type of cDNA library that was used to identify MuSK, that of injured skeletal muscle (Valenzuela et al., 1995).

3) Assuming that the protein that binds to the MuSK NPXY motif resembles Shc, both structurally and functionally, a few experiments could be proposed to study its role during agrin-induced clustering. Presumably, similar experiments would also be feasible when the Shc-like protein will be identified. Expression of various Shc mutants demonstrated that they could block normal Shc function. For example, expression of full length Shc with a mutation in the Grb2 binding site (Y317F) acted as a dominant negative (Ishihara et al., 1997; Stevenson et al., 1999). Expression of partial Shc proteins encoding the PTB domains (Milia et al., 1996; Blaikie et al., 1997) also blocked Shc normal function, presumably by inducing non-productive protein-protein interactions.

Acknowledgements

I would like to thank Drs. David Kaplan and Paul Holland for generously providing the adenoviruses and the C2C12-CAR cells, respectively. I am grateful to Dr Michael Ferns for his constant supply of recombinant agrin and for instructive discussions. I would like to thank my supervisor, Dr. Salvatore Carbonetto, for his support and guidance throughout this project, which was a unique and stimulating learning experience. I cannot forget the precious contribution of all the members of Dr Carbonetto's laboratory, Patrice Côté, Christian Jacobson, Michael Lindenbaum, Federica Montanaro, Hakima Moukhles, Ramin Raouf and Youngen Zhang: your technical expertise and scientific insights were invaluable. I would also like to thank my parents for supporting me while I was writing my thesis. Finally, I am indebted to Lindsay Borthwick, who corrected most of the text and, importantly, who was always an infinite source of inspiration.

Chapter 5

References

Reference list

Altiok N, Altiok S, Changeux JP (1997) Heregulin-stimulated acetylcholine receptor gene expression in muscle: requirement for MAP kinase and evidence for a parallel inhibitory pathway independent of electrical activity. EMBO J 16: 717-725.

Altiok N, Bessereau JL, Changeux JP (1995) ErbB3 and ErbB2/neu mediate the effect of heregulin on acetylcholine receptor gene expression in muscle: differential expression at the endplate. EMBO J 14: 4258-4266.

Anderson MJ, Cohen MW (1977) Nerve-induced and spontaneous redistribution of acetylcholine receptors on cultured muscle cells. J Physiol (Lond) 268: 757-773.

Anthony DT, Schuetze SM, Rubin LL (1984) Transformation by Rous sarcoma virus prevents acetylcholine receptor clustering on cultured chicken muscle fibers. Proc Natl Acad Sci U S A 81: 2265-2269.

Apel ED, Glass DJ, Moscoso LM, Yancopoulos GD, Sanes JR (1997) Rapsyn is required for MuSK signaling and recruits synaptic components to a MuSK-containing scaffold. Neuron 18: 623-635.

Apel ED, Roberds SL, Campbell KP, Merlie JP (1995) Rapsyn may function as a link between the acetylcholine receptor and the agrin-binding dystrophin-associated glycoprotein complex. Neuron 15: 115-126.

Askanas V, Cave S, Gallez-Hawkins G, Engel WK (1985) Fibroblast growth factor, epidermal growth factor and insulin exert a neuronal-like influence on acetylcholine receptors in aneurally cultured human muscle. Neurosci Lett 61: 213-219.

Avruch J, Zhang XF, Kyriakis JM (1994) Raf meets Ras: completing the framework of a signal transduction pathway. Trends Biochem Sci 19: 279-283.

Baker LP, Chen Q, Peng HB (1992) Induction of acetylcholine receptor clustering by native polystyrene beads. Implication of an endogenous muscle-derived signalling system. J Cell Sci 102 (Pt 3): 543-555.

Baker LP, Peng HB (1993) Tyrosine phosphorylation and acetylcholine receptor cluster formation in cultured Xenopus muscle cells. J Cell Biol 120: 185-195.

Baker LP, Peng HB (1995) Induction of acetylcholine receptor cluster formation by local application of growth factors in cultured Xenopus muscle cells. Neurosci Lett 185: 135-138.

Bambrick L, Gordon T (1994) Neurotoxins in the study of neural regulation of membrane proteins in skeletal muscle. J Pharmacol Toxicol Methods 32:129-138.

Barbacci EG, Guarino BC, Stroh JG, Singleton DH, Rosnack KJ, Moyer JD, Andrews GC (1995) The structural basis for the specificity of epidermal growth factor and heregulin binding. J Biol Chem 270: 9585-9589.

Barbacid M (1987) ras genes. Annu Rev Biochem 56: 779-827.

Barnard EA, Dolly JO, Lang B, Lo M, Shorr RG (1979) Application of specifically acting toxins to the detection of functional components common to peripheral and central synapses. Adv Cytopharmacol 3: 409-434.

Batzer AG, Rotin D, Urena JM, Skolnik EY, Schlessinger J (1994) Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor. Mol Cell Biol 14: 5192-5201.

Beitel GJ, Clark SG, Horvitz HR (1990) Caenorhabditis elegans ras gene let-60 acts as a switch in the pathway of vulval induction. Nature 348: 503-509.

Bennett AM, Tonks NK (1997) Regulation of distinct stages of skeletal muscle differentiation by mitogen-activated protein kinases. Science 278: 1288-1291.

Birge RB, Knudsen BS, Besser D, Hanafusa H (1996) SH2 and SH3-containing adaptor proteins: redundant or independent mediators of intracellular signal transduction. Genes Cells 1: 595-613.

Blaikie PA, Fournier E, Dilworth SM, Birnbaum D, Borg JP, Margolis B (1997) The role of the Shc phosphotyrosine interaction/phosphotyrosine binding domain and tyrosine phosphorylation sites in polyoma middle T antigen- mediated cell transformation. J Biol Chem 272: 20671-20677.

Bloch RJ (1983) Acetylcholine receptor clustering in rat myotubes: requirement for CA2+ and effects of drugs which depolymerize microtubules. J Neurosci 3: 2670-2680.

Bloch RJ, Froehner SC (1987) The relationship of the postsynaptic 43K protein to acetylcholine receptors in receptor clusters isolated from cultured rat myotubes. J Cell Biol 104: 645-654.

Boguski MS, McCormick F (1993) Proteins regulating Ras and its relatives. Nature 366: 643-654.

Bonfini L, Migliaccio E, Pelicci G, Lanfrancone L, Pelicci PG (1996) Not all Shc's roads lead to Ras. Trends Biochem Sci 21: 257-261.

Borg JP, Margolis B (1998) Function of PTB domains. Curr Top Microbiol Immunol 228: 23-38.

Borrello MG, Pelicci G, Arighi E, De Filippis L, Greco A, Bongarzone I, Rizzetti M, Pelicci PG, Pierotti MA (1994) The oncogenic versions of the Ret and Trk tyrosine kinases bind Shc and Grb2 adaptor proteins. Oncogene 9: 1661-1668.

Bourne HR, Sanders DA, McCormick F (1990) The GTPase superfamily: a conserved switch for diverse cell functions. Nature 348: 125-132.

Bourne HR, Sanders DA, McCormick F (1991) The GTPase superfamily: conserved structure and molecular mechanism. Nature 349: 117-127.

Bowen DC, Park JS, Bodine S, Stark JL, Valenzuela DM, Stitt TN, Yancopoulos GD, Lindsay RM, Glass DJ, DiStefano PS (1998) Localization and regulation of MuSK at the neuromuscular junction. Dev Biol 199: 309-319.

Brennan C, Scotland PB, Froehner SC, Henderson LP (1992) Functional properties of acetylcholine receptors coexpressed with the 43K protein in heterologous cell systems. Dev Biol 149: 100-111.

Buday L, Downward J (1993) Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. Cell 73: 611-620.

Burden SJ (1998) The formation of neuromuscular synapses. Genes Dev 12: 133-148.

Burgering BM, Pronk GJ, Medema JP, van d, V, Vries Smits AM, van Weeren PC, Bos JL (1993) Role of p21ras in growth factor signal transduction. Biochem Soc Trans 21: 888-894.

Burgess RW, Nguyen QT, Son YJ, Lichtman JW, Sanes JR (1999) Alternatively spliced isoforms of n. Neuron 23: 33-44.

Burkin DJ, Gu M, Hodges BL, Campanelli JT, Kaufman SJ (1998) A functional role for specific spliced variants of the alpha7beta1 integrin in acetylcholine receptor clustering. J Cell Biol 143: 1067-1075.

Cai H, Szeberenyi J, Cooper GM (1990) Effect of a dominant inhibitory Ha-ras mutation on mitogenic signal transduction in NIH 3T3 cells. Mol Cell Biol 10: 5314-5323.

Calera MR, Pilch PF (1998) Induction of Akt-2 correlates with differentiation in Sol8 muscle cells. Biochem Biophys Res Commun 251: 835-841.

Campanelli JT, Roberds SL, Campbell KP, Scheller RH (1994) A role for dystrophin-associated glycoproteins and utrophin in agrin- induced AChR clustering. Cell 77: 663-674.

Carbonetto S, Lindenbaum M (1995) The basement membrane at the neuromuscular junction: a synaptic mediatrix. Curr Opin Neurobiol 5: 596-605.

Caroni P, Rotzler S, Britt JC, Brenner HR (1993) Calcium influx and protein phosphorylation mediate the metabolic stabilization of synaptic acetylcholine receptors in muscle. J Neurosci 13: 1315-1325.

Cartaud A, Coutant S, Petrucci TC, Cartaud J (1998) Evidence for in situ and in vitro association between beta-dystroglycan and the subsynaptic 43K rapsyn protein. Consequence for acetylcholine receptor clustering at the synapse. J Biol Chem 273: 11321-11326.

Cattaneo E, Pelicci PG (1998) Emerging roles for SH2/PTB-containing Shc adaptor proteins in the developing mammalian brain. Trends Neurosci 21: 476-481.

Chamberlain JS, Jaynes JB, Hauschka SD (1985) Regulation of creatine kinase induction in differentiating mouse myoblasts. Mol Cell Biol 5: 484-492.

Chardin P, Camonis JH, Gale NW, Van Aelst L, Schlessinger J, Wigler MH, Bar-Sagi D (1993) Human Sos1: a guanine nucleotide exchange factor for Ras that binds to GRB2. Science 260: 1338-1343.

Charest A, Wagner J, Jacob S, McGlade CJ, Tremblay ML (1996) Phosphotyrosine-independent binding of SHC to the NPLH sequence of murine protein-tyrosine phosphatase-PEST. Evidence for extended phosphotyrosine binding/phosphotyrosine interaction domain recognition specificity. J Biol Chem 271: 8424-8429.

Chen HJ, Rojas-Soto M, Oguni A, Kennedy MB (1998) A synaptic Ras-GTPase activating protein (p135 SynGAP) inhibited by CaM kinase II. Neuron 20: 895-904.

Cherniack AD, Klarlund JK, Conway BR, Czech MP (1995) Disassembly of Son-of-sevenless proteins from Grb2 during p21ras desensitization by insulin. J Biol Chem 270: 1485-1488.

Chiu AY, Ko J (1994) A novel epitope of entactin is present at the mammalian neuromuscular junction. J Neurosci 14: 2809-2817.

Chow JC, Condorelli G, Smith RJ (1998) Insulin-like growth factor-I receptor internalization regulates signaling via the Shc/mitogen-activated protein kinase pathway, but not the insulin receptor substrate-1 pathway. J Biol Chem 273: 4672-4680.

Chow JC, Ling PR, Qu Z, Laviola L, Ciccarone A, Bistrian BR, Smith RJ (1996) Growth hormone stimulates tyrosine phosphorylation of JAK2 and STAT5, but not insulin receptor substrate-1 or SHC proteins in liver and skeletal muscle of normal rats in vivo. Endocrinology 137: 2880-2886.

Chu GC, Moscoso LM, Sliwkowski MX, Merlie JP (1995) Regulation of the acetylcholine receptor epsilon subunit gene by recombinant ARIA: an in vitro model for transynaptic gene regulation. Neuron 14: 329-339.

Cohen I, Rimer M, Lomo T, McMahan UJ (1997) Agrin-induced postsynaptic-like apparatus in skeletal muscle fibers in vivo. Mol Cell Neurosci 9: 237-253.

Colledge M, Froehner SC (1998) Signals mediating ion channel clustering at the neuromuscular junction. Curr Opin Neurobiol 8: 357-363.

Cook SJ, Rubinfeld B, Albert I, McCormick F (1993) RapV12 antagonizes Ras-dependent activation of ERK1 and ERK2 by LPA and EGF in Rat-1 fibroblasts. EMBO J 12: 3475-3485.

Coolican SA, Samuel DS, Ewton DZ, McWade FJ, Florini JR (1997) The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. J Biol Chem 272: 6653-6662.

Corfas G, Falls DL, Fischbach GD (1993) ARIA, a protein that stimulates acetylcholine receptor synthesis, also induces tyrosine phosphorylation of a 185-kDa muscle transmembrane protein. Proc Natl Acad Sci U S A 90: 1624-1628.

Corfas G, Fischbach GD (1993) The number of Na+ channels in cultured chick muscle is increased by ARIA, an acetylcholine receptor-inducing activity. J Neurosci 13: 2118-2125.

Covault J, Sanes JR (1986) Distribution of N-CAM in synaptic and extrasynaptic portions of developing and adult skeletal muscle. J Cell Biol 102: 716-730.

Daggett DF, Cohen MW, Stone D, Nikolics K, Rauvala H, Peng HB (1996) The role of an agrin-growth factor interaction in ACh receptor clustering. Mol Cell Neurosci 8: 272-285.

Dai Z, Peng HB (1992) The influence of basic fibroblast growth factor on acetylcholine receptors in cultured muscle cells. Neurosci Lett 144: 14-18.

Daum G, Eisenmann-Tappe I, Fries HW, Troppmair J, Rapp UR (1994) The ins and outs of Raf kinases. Trends Biochem Sci 19: 474-480.

Davis RJ (1993) The mitogen-activated protein kinase signal transduction pathway. J Biol Chem 268: 14553-14556.

DeChiara TM, Bowen DC, Valenzuela DM, Simmons MV, Poueymirou WT, Thomas S, Kinetz E, Compton DL, Rojas E, Park JS, Smith C, DiStefano PS, Glass DJ, Burden SJ, Yancopoulos GD (1996) The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. Cell 85: 501-512.

Denhardt DT (1996) Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell: the potential for multiplex signalling. Biochem J 318 (Pt 3): 729-747.

Dennis JW, Kosh K, Bryce DM, Breitman ML (1989) Oncogenes conferring metastatic potential induce increased branching of Asn-linked oligosaccharides in rat2 fibroblasts. Oncogene 4: 853-860.

Denzer AJ, Brandenberger R, Gesemann M, Chiquet M, Ruegg MA (1997a) Agrin binds to the nerve-muscle basal lamina via laminin. J Cell Biol 137: 671-683.

Denzer AJ, Gesemann M, Schumacher B, Ruegg MA (1995) An amino-terminal extension is required for the secretion of chick agrin and its binding to extracellular matrix. J Cell Biol 131: 1547-1560.

Denzer AJ, Hauser DM, Gesemann M, Ruegg MA (1997b) Synaptic differentiation: the role of agrin in the formation and maintenance of the neuromuscular junction. Cell Tissue Res 290: 357-365.

Dikic I, Batzer AG, Blaikie P, Obermeier A, Ullrich A, Schlessinger J, Margolis B (1995) Shc binding to nerve growth factor receptor is mediated by the phosphotyrosine interaction domain. J Biol Chem 270: 15125-15129.

Dorman CM, Johnson SE (1999) Activated Raf inhibits avian myogenesis through a MAPK-dependent mechanism. Oncogene 18: 5167-5176.

Duca KA, Chiu KP, Sullivan T, Berman SA, Bursztajn S (1998) Nuclear clustering in myotubes: a proposed role in acetylcholine receptor mRNA expression. Biochim Biophys Acta 1401: 1-20.

Ewton DZ, Coolican SA, Mohan S, Chernausek SD, Florini JR (1998) Modulation of insulin-like growth factor actions in L6A1 myoblasts by insulin-like growth factor binding protein (IGFBP)-4 and IGFBP-5: a dual role for IGFBP-5. J Cell Physiol 177: 47-57.

Fallon JR, Hall ZW (1994) Building synapses: agrin and dystroglycan stick together. Trends Neurosci 17: 469-473.

Falls DL, Rosen KM, Corfas G, Lane WS, Fischbach GD (1993) ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. Cell 72: 801-815.

Feig LA, Cooper GM (1988) Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. Mol Cell Biol 8: 3235-3243.

Ferns M, Deiner M, Hall Z (1996) Agrin-induced acetylcholine receptor clustering in mammalian muscle requires tyrosine phosphorylation. J Cell Biol 132: 937-944.

Ferns M, Hoch W, Campanelli JT, Rupp F, Hall ZW, Scheller RH (1992) RNA splicing regulates agrin-mediated acetylcholine receptor clustering activity on cultured myotubes. Neuron 8: 1079-1086.

Ferns MJ, Campanelli JT, Hoch W, Scheller RH, Hall Z (1993) The ability of agrin to cluster AChRs depends on alternative splicing and on cell surface proteoglycans. Neuron 11: 491-502.

Florini JR, Samuel DS, Ewton DZ, Kirk C, Sklar RM (1996) Stimulation of myogenic differentiation by a neuregulin, glial growth factor 2. Are neuregulins the long-sought muscle trophic factors secreted by nerves? J Biol Chem 271: 12699-12702.

Flucher BE, Daniels MP (1989) Distribution of Na+ channels and ankyrin in neuromuscular junctions is complementary to that of acetylcholine receptors and the 43 kd protein. Neuron 3: 163-175.

Frail DE, McLaughlin LL, Mudd J, Merlie JP (1988) Identification of the mouse muscle 43,000-dalton acetylcholine receptor- associated protein (RAPsyn) by cDNA cloning. J Biol Chem 263: 15602-15607.

Froehner SC, Adams ME, Peters MF, Gee SH (1997) Syntrophins: modular adapter proteins at the neuromuscular junction and the sarcolemma. Soc Gen Physiol Ser 52: 197-207.

Froehner SC, Gulbrandsen V, Hyman C, Jeng AY, Neubig RR, Cohen JB (1981) Immunofluorescence localization at the mammalian neuromuscular junction of the Mr 43,000 protein of Torpedo postsynaptic membranes. Proc Natl Acad Sci U S A 78: 5230-5234.

Fuhrer C, Hall ZW (1996) Functional interaction of Src family kinases with the acetylcholine receptor in C2 myotubes. J Biol Chem 271: 32474-32481.

Fukami Y, Owada MK, Sumi M, Hayashi F (1986) A p60v-src-related tyrosine kinase in the acetylcholine receptor-rich membranes of Narke japonica: association and dissociation of phosphatidylinositol kinase activity. Biochem Biophys Res Commun 139: 473-479.

Gallo R, Serafini M, Castellani L, Falcone G, Alema S (1999) Distinct effects of rac1 on differentiation of primary avian myoblasts. Mol Biol Cell 10: 3137-3150.

Gautam M, DeChiara TM, Glass DJ, Yancopoulos GD, Sanes JR (1999) Distinct phenotypes of mutant mice lacking agrin, MuSK, or rapsyn. Brain Res Dev Brain Res 114: 171-178.

Gautam M, Noakes PG, Moscoso L, Rupp F, Scheller RH, Merlie JP, Sanes JR (1996) Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. Cell 85: 525-535.

Gautam M, Noakes PG, Mudd J, Nichol M, Chu GC, Sanes JR, Merlie JP (1995) Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. Nature 377: 232-236.

Gawler DJ (1998) Points of convergence between Ca2+ and Ras signalling pathways. Biochim Biophys Acta 1448: 171-182.

Gee SH, Montanaro F, Lindenbaum MH, Carbonetto S (1994) Dystroglycan-alpha, a dystrophin-associated glycoprotein, is a functional agrin receptor. Cell 77: 675-686.

Gesemann M, Cavalli V, Denzer AJ, Brancaccio A, Schumacher B, Ruegg MA (1996) Alternative splicing of agrin alters its binding to heparin, dystroglycan, and the putative agrin receptor. Neuron 16: 755-767.

Gesemann M, Denzer AJ, Ruegg MA (1995) Acetylcholine receptor-aggregating activity of agrin isoforms and mapping of the active site. J Cell Biol 128: 625-636.

Gillespie SK, Balasubramanian S, Fung ET, Huganir RL (1996) Rapsyn clusters and activates the synapse-specific receptor tyrosine kinase MuSK. Neuron 16: 953-962.

Glass DJ, Apel ED, Shah S, Bowen DC, DeChiara TM, Stitt TN, Sanes JR, Yancopoulos GD (1997) Kinase domain of the muscle-specific receptor tyrosine kinase (MuSK) is sufficient for phosphorylation but not clustering of acetylcholine receptors: required role for the MuSK ectodomain? Proc Natl Acad Sci U S A 94: 8848-8853.

Glass DJ, Bowen DC, Stitt TN, Radziejewski C, Bruno J, Ryan TE, Gies DR, Shah S, Mattsson K, Burden SJ, DiStefano PS, Valenzuela DM, DeChiara TM, Yancopoulos GD (1996c) Agrin acts via a MuSK receptor complex. Cell 85: 513-523.

Glass DJ, Bowen DC, Stitt TN, Radziejewski C, Bruno J, Ryan TE, Gies DR, Shah S, Mattsson K, Burden SJ, DiStefano PS, Valenzuela DM, DeChiara TM, Yancopoulos GD (1996a) Agrin acts via a MuSK receptor complex. Cell 85: 513-523.

Glass DJ, DeChiara TM, Stitt TN, DiStefano PS, Valenzuela DM, Yancopoulos GD (1996b) The receptor tyrosine kinase MuSK is required for neuromuscular junction formation and is a functional receptor for agrin. Cold Spring Harb Symp Quant Biol 61: 435-444.

Glass DJ, Yancopoulos GD (1997) Sequential roles of agrin, MuSK and rapsyn during neuromuscular junction formation. Curr Opin Neurobiol 7: 379-384.

Godfrey EW, Nitkin RM, Wallace BG, Rubin LL, McMahan UJ (1984) Components of Torpedo electric organ and muscle that cause aggregation of acetylcholine receptors on cultured muscle cells. J Cell Biol 99: 615-627.

Gonzalez M, Ruggiero FP, Chang Q, Shi YJ, Rich MM, Kraner S, Balice-Gordon RJ (1999) Disruption of Trkb-mediated signaling induces disassembly of postsynaptic receptor clusters at neuromuscular junctions. Neuron 24: 567-583.

Goodearl AD, Yee AG, Sandrock AW, Jr., Corfas G, Fischbach GD (1995) ARIA is concentrated in the synaptic basal lamina of the developing chick neuromuscular junction. J Cell Biol 130: 1423-1434.

Gossett LA, Zhang W, Olson EN (1988) Dexamethasone-dependent inhibition of differentiation of C2 myoblasts bearing steroid-inducible N-ras oncogenes. J Cell Biol 106: 2127-2137.

Gramolini AO, Angus LM, Schaeffer L, Burton EA, Tinsley JM, Davies KE, Changeux JP, Jasmin BJ (1999) Induction of utrophin gene expression by heregulin in skeletal muscle cells: role of the N-box motif and GA binding protein. Proc Natl Acad Sci U S A 96: 3223-3227.

Grow WA, Ferns M, Gordon H (1999) Agrin-independent activation of the agrin signal transduction pathway. J Neurobiol 40: 356-365.

Gu Y, Hall ZW (1988) Characterization of acetylcholine receptor subunits in developing and in denervated mammalian muscle. J Biol Chem 263: 12878-12885.

Hall ZW, Sanes JR (1993) Synaptic structure and development: the neuromuscular junction. Cell 72 Suppl: 99-121.

Hallberg B, Ashcroft M, Loeb DM, Kaplan DR, Downward J (1998) Nerve growth factor induced stimulation of Ras requires Trk interaction with Shc but does not involve phosphoinositide 3-OH kinase. Oncogene 17: 691-697.

Han B, Fischbach GD (1999b) Processing of ARIA and release from isolated nerve terminals. Philos Trans R Soc Lond B Biol Sci 354: 411-416.

Han B, Fischbach GD (1999a) The release of acetylcholine receptor inducing activity (ARIA) from its transmembrane precursor in transfected fibroblasts. J Biol Chem 274: 26407-26415.

Han M, Sternberg PW (1990) let-60, a gene that specifies cell fates during C. elegans vulval induction, encodes a ras protein. Cell 63: 921-931.

He W, O'Neill TJ, Gustafson TA (1995) Distinct modes of interaction of SHC and insulin receptor substrate-1 with the insulin receptor NPEY region via non-SH2 domains. J Biol Chem 270: 23258-23262.

Henry MD, Williamson RA, Campbell KP (1998) Analysis of the role of dystroglycan in early postimplantation mouse development. Ann N Y Acad Sci 857: 256-259.

Hilgenberg L, Miles K (1995) Developmental regulation of a protein kinase C isoform localized in the neuromuscular junction. J Cell Sci 108 (Pt 1): 51-61.

Hoch W, Campanelli JT, Harrison S, Scheller RH (1994) Structural domains of agrin required for clustering of nicotinic acetylcholine receptors. EMBO J 13: 2814-2821.

Hoch W, Ferns M, Campanelli JT, Hall ZW, Scheller RH (1993) Developmental regulation of highly active alternatively spliced forms of agrin. Neuron 11: 479-490.

Hopf C, Hoch W (1997) Heparin inhibits acetylcholine receptor aggregation at two distinct steps in the agrin-induced pathway. Eur J Neurosci 9: 1170-1177.

Hopf C, Hoch W (1998) Tyrosine phosphorylation of the muscle-specific kinase is exclusively induced by acetylcholine receptor-aggregating agrin fragments. Eur J Biochem 253: 382-389.

Hopfield JF, Tank DW, Greengard P, Huganir RL (1988) Functional modulation of the nicotinic acetylcholine receptor by tyrosine phosphorylation. Nature 336: 677-680.

Huang C, Ni Y, Wang T, Gao Y, Haudenschild CC, Zhan X (1997) Down-regulation of the filamentous actin cross-linking activity of cortactin by Src-mediated tyrosine phosphorylation. J Biol Chem 272: 13911-13915.

Hughes PE, Renshaw MW, Pfaff M, Forsyth J, Keivens VM, Schwartz MA, Ginsberg MH (1997) Suppression of integrin activation: a novel function of a Ras/Raf- initiated MAP kinase pathway. Cell 88: 521-530.

Isakoff SJ, Yu YP, Su YC, Blaikie P, Yajnik V, Rose E, Weidner KM, Sachs M, Margolis B, Skolnik EY (1996) Interaction between the phosphotyrosine binding domain of Shc

and the insulin receptor is required for Shc phosphorylation by insulin in vivo. J Biol Chem 271: 3959-3962.

Ishihara H, Sasaoka T, Ishiki M, Takata Y, Imamura T, Usui I, Langlois WJ, Sawa T, Kobayashi M (1997) Functional importance of Shc tyrosine 317 on insulin signaling in Rat1 fibroblasts expressing insulin receptors. J Biol Chem 272: 9581-9586.

Jennings CG, Burden SJ (1993) Development of the neuromuscular synapse. Curr Opin Neurobiol 3: 75-81.

Jennings CG, Mudge AW (1989) Chick myotubes in culture express high-affinity receptors for calcitonin gene-related peptide. Brain Res 504: 199-205.

Jiang BH, Aoki M, Zheng JZ, Li J, Vogt PK (1999) Myogenic signaling of phosphatidylinositol 3-kinase requires the serine- threonine kinase Akt/protein kinase B. Proc Natl Acad Sci U S A 96: 2077-2081.

Jiang BH, Zheng JZ, Vogt PK (1998) An essential role of phosphatidylinositol 3-kinase in myogenic differentiation. Proc Natl Acad Sci U S A 95: 14179-14183.

Jo SA, Zhu X, Marchionni MA, Burden SJ (1995) Neuregulins are concentrated at nerve-muscle synapses and activate ACh- receptor gene expression. Nature 373: 158-161.

Jones G, Herczeg A, Ruegg MA, Lichtsteiner M, Kroger S, Brenner HR (1996) Substrate-bound agrin induces expression of acetylcholine receptor epsilon-subunit gene in cultured mammalian muscle cells. Proc Natl Acad Sci U S A 93: 5985-5990.

Jones G, Meier T, Lichtsteiner M, Witzemann V, Sakmann B, Brenner HR (1997) Induction by agrin of ectopic and functional postsynaptic-like membrane in innervated muscle. Proc Natl Acad Sci U S A 94: 2654-2659.

Jones G, Moore C, Hashemolhosseini S, Brenner HR (1999) Constitutively active MuSK is clustered in the absence of agrin and induces ectopic postsynaptic-like membranes in skeletal muscle fibers. J Neurosci 19: 3376-3383.

Joneson T, White MA, Wigler MH, Bar-Sagi D (1996) Stimulation of membrane ruffling and MAP kinase activation by distinct effectors of RAS. Science 271: 810-812.

Kaliman P, Vinals F, Testar X, Palacin M, Zorzano A (1996) Phosphatidylinositol 3-kinase inhibitors block differentiation of skeletal muscle cells. J Biol Chem 271: 19146-19151.

Kaplan DR (1998) Studying signal transduction in neuronal cells: the Trk/NGF system. Prog Brain Res 117: 35-46.

Kasus-Jacobi A, Perdereau D, Tartare-Deckert S, Van Obberghen E, Girard J, Burnol AF (1997) Evidence for a direct interaction between insulin receptor substrate-1 and Shc. J Biol Chem 272: 17166-17170.

Kavanaugh WM, Turck CW, Williams LT (1995) PTB domain binding to signaling proteins through a sequence motif containing phosphotyrosine. Science 268: 1177-1179.

Kavanaugh WM, Williams LT (1994) An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. Science 266: 1862-1865.

Kawachi H, Tamura H, Watakabe I, Shintani T, Maeda N, Noda M (1999) Protein tyrosine phosphatase zeta/RPTPbeta interacts with PSD-95/SAP90 family. Brain Res Mol Brain Res 72: 47-54.

Khosravi-Far R, White MA, Westwick JK, Solski PA, Chrzanowska-Wodnicka M, Van Aelst L, Wigler MH, Der CJ (1996) Oncogenic Ras activation of Raf/mitogen-activated protein kinase- independent pathways is sufficient to cause tumorigenic transformation. Mol Cell Biol 16: 3923-3933.

Kikuchi A, Demo SD, Ye ZH, Chen YW, Williams LT (1994) ralGDS family members interact with the effector loop of ras p21. Mol Cell Biol 14: 7483-7491.

Kim JH, Liao D, Lau LF, Huganir RL (1998) SynGAP: a synaptic RasGAP that associates with the PSD-95/SAP90 protein family. Neuron 20: 683-691.

Kim D, Chi S, Lee KH, Rhee S, Kwon YK, Chung CH, Kwon H, Kang MS (1999) Neuregulin stimulates myogenic differentiation in an autocrine manner. J Biol Chem 274: 15395-15400.

Kinnunen T, Kaksonen M, Saarinen J, Kalkkinen N, Peng HB, Rauvala H (1998) Cortactin-Src kinase signaling pathway is involved in N-syndecan- dependent neurite outgrowth. J Biol Chem 273: 10702-10708.

Klarsfeld A, Bessereau JL, Salmon AM, Triller A, Babinet C, Changeux JP (1991) An acetylcholine receptor alpha-subunit promoter conferring preferential synaptic expression in muscle of transgenic mice. EMBO J 10: 625-632.

Klesse LJ, Parada LF (1999) Trks: signal transduction and intracellular pathways. Microsc Res Tech 45: 210-216.

Konieczny SF, Drobes BL, Menke SL, Taparowsky EJ (1989) Inhibition of myogenic differentiation by the H-ras oncogene is associated with the down regulation of the MyoD1 gene. Oncogene 4: 473-481.

Krikorian JG, Daniels MP (1989) Reorganization and stabilization of acetylcholine receptor aggregates on rat myotubes. Dev Biol 131: 524-538.

Kudla AJ, Jones NC, Rosenthal RS, Arthur K, Clase KL, Olwin BB (1998) The FGF receptor-1 tyrosine kinase domain regulates myogenesis but is not sufficient to stimulate proliferation. J Cell Biol 142: 241-250.

Laird AD, Morrison DK, Shalloway D (1999) Characterization of Raf-1 activation in mitosis. J Biol Chem 274:4430-4439.

Laminet AA, Apell G, Conroy L, Kavanaugh WM (1996) Affinity, specificity, and kinetics of the interaction of the SHC phosphotyrosine binding domain with asparagine-X-X-phosphotyrosine motifs of growth factor receptors. J Biol Chem 271: 264-269.

Lamkin TD, Walk SF, Liu L, Damen JE, Krystal G, Ravichandran KS (1997) Shc interaction with Src homology 2 domain containing inositol phosphatase (SHIP) in vivo requires the Shc-phosphotyrosine binding domain and two specific phosphotyrosines on SHIP. J Biol Chem 272: 10396-10401.

Lau LF, Mammen A, Ehlers MD, Kindler S, Chung WJ, Garner CC, Huganir RL (1996) Interaction of the N-methyl-D-aspartate receptor complex with a novel synapse-associated protein, SAP102. J Biol Chem 271: 21622-21628.

Lauri SE, Kaukinen S, Kinnunen T, Ylinen A, Imai S, Kaila K, Taira T, Rauvala H (1999) Reglulatory role and molecular interactions of a cell-surface heparan sulfate proteoglycan (N-syndecan) in hippocampal long-term potentiation. J Neurosci 19: 1226-1235.

Lawrence JB, Taneja K, Singer RH (1989) Temporal resolution and sequential expression of muscle-specific genes revealed by in situ hybridization. Dev Biol 133: 235-246.

Lemke G (1996) Neuregulins in development. Mol Cell Neurosci 7: 247-262.

Leevers SJ, Paterson HF, Marshall CJ (1994) Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. Nature 369: 411-414.

Li N, Batzer A, Daly R, Yajnik V, Skolnik E, Chardin P, Bar-Sagi D, Margolis B, Schlessinger J (1993) Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. Nature 363: 85-88.

Lin K, Abraham KM (1997) Targets of p56(lck) activity in immature thymoblasts: stimulation of the Ras/Raf/MAPK pathway. Int Immunol 9: 291-306.

Lin JH, Makris A, McMahon C, Bear SE, Patriotis C, Prasad VR, Brent R, Golemis EA, Tsichlis PN (1999) The ankyrin repeat-containing adaptor protein Tvl-1 is a novel substrate and regulator of Raf-1. J.Biol.Chem. 274:14706-14715.

Loeb JA, Susanto ET, Fischbach GD (1998) The neuregulin precursor proARIA is processed to ARIA after expression on the cell surface by a protein kinase C-enhanced mechanism. Mol Cell Neurosci 11: 77-91.

Lowenstein EJ, Daly RJ, Batzer AG, Li W, Margolis B, Lammers R, Ullrich A, Skolnik EY, Bar-Sagi D, Schlessinger J (1992) The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. Cell 70: 431-442.

Lu JT, Son YJ, Lee J, Jetton TL, Shiota M, Moscoso L, Niswender KD, Loewy AD, Magnuson MA, Sanes JR, Emeson RB (1999) Mice lacking alpha-calcitonin gene-related peptide exhibit normal cardiovascular regulation and neuromuscular development. Mol Cell Neurosci 14: 99-120.

Ma J, Nastuk MA, McKechnie BA, Fallon JR (1993) The agrin receptor. Localization in the postsynaptic membrane, interaction with agrin, and relationship to the acetylcholine receptor. J Biol Chem 268: 25108-25117.

Macara IG, Lounsbury KM, Richards SA, McKiernan C, Bar-Sagi D (1996) The Ras superfamily of GTPases. FASEB J 10: 625-630.

Maeda N, Ichihara-Tanaka K, Kimura T, Kadomatsu K, Muramatsu T, Noda M (1999) A receptor-like protein-tyrosine phosphatase PTPzeta/RPTPbeta binds a heparin-binding growth factor midkine. Involvement of arginine 78 of midkine in the high affinity binding to PTPzeta. J Biol Chem 274: 12474-12479.

Majewski M, Nieborowska-Skorska M, Salomoni P, Slupianek A, Reiss K, Trotta R, Calabretta B, Skorski T (1999) Activation of mitochondrial Raf-1 is involved in the antiapoptotic effects of Akt. Cancer Res 59: 2815-2819.

Manchester J, Kong X, Lowry OH, Lawrence JC Jr (1994) Ras signaling in the activation of glucose transport by insulin. PNAS 91: 4644-4648.

Marais R, Marshall CJ (1996) Control of the ERK MAP kinase cascade by Ras and Raf. Cancer Surv 27:101-125

Martin PT, Kaufman SJ, Kramer RH, Sanes JR (1996) Synaptic integrins in developing, adult, and mutant muscle: selective association of alpha1, alpha7A, and alpha7B integrins with the neuromuscular junction. Dev Biol 174: 125-139.

Martin PT, Sanes JR (1995) Role for a synapse-specific carbohydrate in agrin-induced clustering of acetylcholine receptors. Neuron 14: 743-754.

Martin PT, Sanes JR (1997) Integrins mediate adhesion to agrin and modulate agrin signaling. Development 124: 3909-3917.

Martin PT, Scott LJ, Porter BE, Sanes JR (1999) Distinct structures and functions of related pre- and postsynaptic carbohydrates at the mammalian neuromuscular junction. Mol Cell Neurosci 13: 105-118.

Masiakowski P, Carroll RD (1992) A novel family of cell surface receptors with tyrosine kinase-like domain. J Biol Chem 267: 26181-26190.

Mayer U, Saher G, Fassler R, Bornemann A, Echtermeyer F, von der Mark H, Miosge N, Poschl E, von der Mark K (1997) Absence of integrin alpha 7 causes a novel form of muscular dystrophy. Nat Genet 17: 318-323.

Mazzoni IE, Said FA, Aloyz R, Miller FD, Kaplan D (1999) Ras regulates sympathetic neuron survival by suppressing the p53- mediated cell death. J Neurosci 19: 9716-9727.

Megeath LJ, Fallon JR (1998) Intracellular calcium regulates agrin-induced acetylcholine receptor clustering. J Neurosci 18: 672-678.

Meier T, Gesemann M, Cavalli V, Ruegg MA, Wallace BG (1996) AChR phosphorylation and aggregation induced by an agrin fragment that lacks the binding domain for alpha-dystroglycan. EMBO J 15: 2625-2631.

Meier T, Hauser DM, Chiquet M, Landmann L, Ruegg MA, Brenner HR (1997) Neural agrin induces ectopic postsynaptic specializations in innervated muscle fibers. J Neurosci 17: 6534-6544.

Meier T, Marangi PA, Moll J, Hauser DM, Brenner HR, Ruegg MA (1998b) A minigene of neural agrin encoding the laminin-binding and acetylcholine receptor-aggregating domains is sufficient to induce postsynaptic differentiation in muscle fibres. Eur J Neurosci 10: 3141-3152.

Meier T, Perez GM, Wallace BG (1995) Immobilization of nicotinic acetylcholine receptors in mouse C2 myotubes by agrin-induced protein tyrosine phosphorylation. J Cell Biol 131: 441-451.

Meier T, Ruegg MA, Wallace BG (1998a) Muscle-specific agrin isoforms reduce phosphorylation of AChR gamma and delta subunits in cultured muscle cells. Mol Cell Neurosci 11: 206-216.

Meyer D, Birchmeier C (1995) Multiple essential functions of neuregulin in development. Nature 378: 386-390.

Meyer G, Wallace BG (1998) Recruitment of a nicotinic acetylcholine receptor mutant lacking cytoplasmic tyrosine residues in its beta subunit into agrin-induced aggregates.

Mol Cell Neurosci 11: 324-333.

Milburn MV, Tong L, deVos AM, Brunger A, Yamaizumi Z, Nishimura S, Kim SH (1990) Molecular switch for signal transduction: structural differences between active and inactive forms of protooncogenic ras proteins. Science 247: 939-945.

Milev P, Chiba A, Haring M, Rauvala H, Schachner M, Ranscht B, Margolis RK, Margolis RU (1998) High affinity binding and overlapping localization of neurocan and phosphacan/protein-tyrosine phosphatase-zeta/beta with tenascin-R, amphoterin, and the heparin-binding growth-associated molecule. J Biol Chem 273: 6998-7005.

Milia E, Di Somma MM, Baldoni F, Chiari R, Lanfrancone L, Pelicci PG, Telford JL, Baldari CT (1996) The aminoterminal phosphotyrosine binding domain of Shc associates with ZAP-70 and mediates TCR dependent gene activation. Oncogene 13: 767-775.

Minichiello L, Casagranda F, Tatche RS, Stucky CL, Postigo A, Lewin GR, Davies AM, Klein R (1998) Point mutation in trkB causes loss of NT4-dependent neurons without major effects on diverse BDNF responses. Neuron 21: 335-345.

Mishina M, Takai T, Imoto K, Noda M, Takahashi T, Numa S, Methfessel C, Sakmann B (1986) Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature 321: 406-411.

Missias AC, Chu GC, Klocke BJ, Sanes JR, Merlie JP (1996) Maturation of the acetylcholine receptor in skeletal muscle: regulation of the AChR gamma-to-epsilon switch. Dev Biol 179: 223-238.

Mohamed AS, Swope SL (1999) Phosphorylation and cytoskeletal anchoring of the acetylcholine receptor by Src class protein-tyrosine kinases. Activation by rapsyn. J Biol Chem 274: 20529-20539.

Montanaro F, Gee SH, Jacobson C, Lindenbaum MH, Froehner SC, Carbonetto S (1998) Laminin and alpha-dystroglycan mediate acetylcholine receptor aggregation via a MuSK-independent pathway. J Neurosci 18: 1250-1260.

Mook-Jung I, Gordon H (1995) Acetylcholine receptor clustering in C2 muscle cells requires chondroitin sulfate. J Neurobiol 28: 482-492.

Morrison DK (1995) Mechanisms regulating Raf-1 activity in signal transduction pathways. Mol Reprod Dev 42: 507-514.

Moscoso LM, Chu GC, Gautam M, Noakes PG, Merlie JP, Sanes JR (1995) Synapse-associated expression of an acetylcholine receptor-inducing protein, ARIA/heregulin, and its putative receptors, ErbB2 and ErbB3, in developing mammalian muscle. Dev Biol 172: 158-169.

Nakagawa H, Mutoh T, Kumano T, Kuriyama M (1998) HMG-CoA reductase inhibitor-induced L6 myoblast cell death: involvement of the phosphatidylinositol 3-kinase pathway. FEBS Lett 438:289-292.

Nalbantoglu J, Pari G, Karpati G, Holland PC (1999) Expression of the primary coxsackie and adenovirus receptor is downregulated during skeletal muscle maturation and limits the efficacy of adenovirus-mediated gene delivery to muscle cells. Hum Gene Ther 10: 1009-1019.

Nancy V, Wolthuis RM, de Tand MF, Janoueix-Lerosey I, Bos JL, de Gunzburg J (1999) Identification and characterization of potential effector molecules of the Ras-related GTPase Rap2. J Biol Chem 274: 8737-8745.

Nastuk MA, Lieth E, Ma JY, Cardasis CA, Moynihan EB, McKechnie BA, Fallon JR (1991) The putative agrin receptor binds ligand in a calcium-dependent manner and aggregates during agrin-induced acetylcholine receptor clustering. Neuron 7: 807-818.

Neubig RR, Krodel EK, Boyd ND, Cohen JB (1979) Acetylcholine and local anesthetic binding to Torpedo nicotinic postsynaptic membranes after removal of nonreceptor peptides. Proc Natl Acad Sci U S A 76: 690-694.

New HV, Mudge AW (1986) Calcitonin gene-related peptide regulates muscle acetylcholine receptor synthesis. Nature 323: 809-811.

Ng YP, Pun S, Yang JF, Ip NY, Tsim KW (1997) Chick muscle expresses various ARIA isoforms: regulation during development, denervation, and regeneration. Mol Cell Neurosci 9: 132-143.

Nimnual AS, Yatsula BA, Bar-Sagi D (1998) Coupling of Ras and Rac guanosine triphosphatases through the Ras exchanger Sos. Science 279: 560-563.

Ohtsuka M, Ihara S, Ogawa R, Watanabe T, Watanabe Y (1984) Preparation and characterization of antibodies to O-phosphotyrosine and their use for identification of phosphotyrosine-containing proteins. Int J Cancer 34: 855-861.

Okabayashi Y, Sugimoto Y, Totty NF, Hsuan J, Kido Y, Sakaguchi K, Gout I, Waterfield MD, Kasuga M (1996) Interaction of Shc with adaptor protein adaptins. J Biol Chem 271: 5265-5269.

Okada S, Kao AW, Ceresa BP, Blaikie P, Margolis B, Pessin JE (1997) The 66-kDa Shc isoform is a negative regulator of the epidermal growth factor-stimulated mitogen-activated protein kinase pathway. J Biol Chem 272: 28042-28049.

Olek AJ, Krikorian JG, Daniels MP (1986) Early stages in the formation and stabilization of acetylcholine receptor aggregates on cultured myotubes: sensitivity to temperature and azide. Dev Biol 17: 24-34.

Olivier JP, Raabe T, Henkemeyer M, Dickson B, Mbamalu G, Margolis B, Schlessinger J, Hafen E, Pawson T (1993) A Drosophila SH2-SH3 adaptor protein implicated in

coupling the sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchange, Sos. Cell 73: 179-191.

Olofsson B (1999) Rho guanine dissociation inhibitors: pivotal molecules in cellular signalling. Cell Signal 11: 545-554.

Olson EN, Spizz G, Tainsky MA (1987) The oncogenic forms of N-ras or H-ras prevent skeletal myoblast differentiation. Mol Cell Biol 7: 2104-2111.

Olson EN, Sternberg E, Hu JS, Spizz G, Wilcox C (1986) Regulation of myogenic differentiation by type beta transforming growth factor. J Cell Biol 103: 1799-1805.

Osterlund M, Fontaine B, Devillers-Thiery A, Geoffroy B, Changeux JP (1989) Acetylcholine receptor expression in primary cultures of embryonic chick myotubes--I. Discoordinate regulation of alpha-g. Neuroscience 32: 279-287.

Paez-Espinosa V, Carvalho CR, Alvarez-Rojas F, Janeri L, Velloso LA, Boschero AC, Saad MJ (1998) Insulin induces tyrosine phosphorylation of Shc and stimulates Shc/GRB2 association in insulin-sensitive tissues of the intact rat. Endocrine 8: 193-200.

Pai EF, Kabsch W, Krengel U, Holmes KC, John J, Wittinghofer A (1989) Structure of the guanine-nucleotide-binding domain of the Ha-ras oncogene product p21 in the triphosphate conformation. Nature 341: 209-214.

Parrini MC, Giglione C, Parmeggiani A (1997) Co-ordination and specificity of the action of GTPase-activating proteins and GDP/GTP-exchange factors on Ras. Biochem Soc Trans 25: 997-1001.

Patton BL, Miner JH, Chiu AY, Sanes JR (1997) Distribution and function of laminins in the neuromuscular system of developing, adult, and mutant mice. J Cell Biol 139: 1507-1521.

Pawson T (1994) Regulation of the Ras signalling pathway by protein-tyrosine kinases. Biochem Soc Trans 22: 455-460.

Payne PA, Olson EN, Hsiau P, Roberts R, Perryman MB, Schneider MD (1987) An activated c-Ha-ras allele blocks the induction of muscle-specific genes whose expression is contingent on mitogen withdrawal. Proc Natl Acad Sci U S A 84: 8956-8960.

Pelicci G, Lanfrancone L, Grignani F, McGlade J, Cavallo F, Forni G, Nicoletti I, Grignani F, Pawson T, Pelicci PG (1992) A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. Cell 70: 93-104.

Pelicci G, Lanfrancone L, Salcini AE, Romano A, Mele S, Grazia BM, Segatto O, Di Fiore PP, Pelicci PG (1995) Constitutive phosphorylation of Shc proteins in human tumors. Oncogene 11: 899-907.

Peng HB, Ali AA, Dai Z, Daggett DF, Raulo E, Rauvala H (1995) The role of heparin-binding growth-associated molecule (HB-GAM) in the postsynaptic induction in cultured muscle cells. J Neurosci 15: 3027-3038.

Peng HB, Baker LP, Chen Q (1991) Induction of synaptic development in cultured muscle cells by basic fibroblast growth factor. Neuron 6: 237-246.

Peng HB, Xie H, Dai Z (1997) Association of cortactin with developing neuromuscular specializations. J Neurocytol 26: 637-650.

Peters MF, Sadoulet-Puccio HM, Grady MR, Kramarcy NR, Kunkel LM, Sanes JR, Sealock R, Froehner SC (1998) Differential membrane localization and intermolecular associations of alpha-dystrobrevin isoforms in skeletal muscle. J Cell Biol 142: 1269-1278.

Pizon V, Cifuentes-Diaz C, Mege RM, Baldacci G, Rieger F (1996) Expression and localization of RAP1 proteins during myogenic differentiation. Eur J Cell Biol 69: 224-235.

Polakis P, McCormick F (1992) Interactions between p21ras proteins and their GTPase activating proteins. Cancer Surv 12: 25-42.

Ponzetto C (1998) Physiological function of receptor-SH2 interactions. Curr Top Microbiol Immunol 228: 165-177.

Prigent SA, Gullick WJ (1994) Identification of c-erbB-3 binding sites for phosphatidylinositol 3'- kinase and SHC using an EGF receptor/c-erbB-3 chimera. EMBO J 13: 2831-2841.

Qiu RG, Chen J, Kirn D, McCormick F, Symons M (1995) An essential role for Rac in Ras transformation. Nature 374: 457-459.

Qu Z, Apel ED, Doherty CA, Hoffman PW, Huganir RL (1996) The Synapse-Associated Protein Rapsyn Regulates Tyrosine Phosphorylation of Proteins Colocalized at Nicotinic Acetylcholine Receptor Clusters. Mol Cell Neurosci 8: 171-184.

Qu Z, Huganir RL (1994) Comparison of innervation and agrin-induced tyrosine phosphorylation of the nicotinic acetylcholine receptor. J Neurosci 14: 6834-6841.

Qu ZC, Moritz E, Huganir RL (1990) Regulation of tyrosine phosphorylation of the nicotinic acetylcholine receptor at the rat neuromuscular junction. Neuron 4: 367-378.

Quilliam LA, Khosravi-Far R, Huff SY, Der CJ (1995) Guanine nucleotide exchange factors: activators of the Ras superfamily of proteins. Bioessays 17: 395-404.

Ramocki MB, Johnson SE, White MA, Ashendel CL, Konieczny SF, Taparowsky EJ (1997) Signaling through mitogen-activated protein kinase and Rac/Rho does not

duplicate the effects of activated Ras on skeletal myogenesis. Mol Cell Biol 17: 3547-3555.

Ramocki MB, White MA, Konieczny SF, Taparowsky EJ (1998) A role for RalGDS and a novel Ras effector in the Ras-mediated inhibition of skeletal myogenesis. J Biol Chem 273: 17696-17701.

Reist NE, Werle MJ, McMahan UJ (1992) Agrin released by motor neurons induces the aggregation of acetylcholine receptors at neuromuscular junctions. Neuron 8: 865-868.

Ridley AJ, Hall A (1992) Distinct patterns of actin organization regulated by the small GTP- binding proteins Rac and Rho. Cold Spring Harb Symp Quant Biol 57: 661-671.

Riethmacher D, Sonnenberg-Riethmacher E, Brinkmann V, Yamaai T, Lewin GR, Birchmeier C (1997) Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. Nature 389: 725-730.

Rimer M, Cohen I, Lomo T, Burden SJ, McMahan UJ (1998) Neuregulins and erbB receptors at neuromuscular junctions and at agrin- induced postsynaptic-like apparatus in skeletal muscle. Mol Cell Neurosci 12: 1-15.

Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD, Downward J (1994) Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 370: 527-532.

Rodriguez-Viciana P, Warne PH, Khwaja A, Marte BM, Pappin D, Das P, Waterfield MD, Ridley A, Downward J (1997) Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. Cell 89: 457-467.

Rommel C, Hafen E (1998) Ras--a versatile cellular switch. Curr Opin Genet Dev 8: 412-418.

Rosenthal N (1989) Muscle cell differentiation. Curr Opin Cell Biol 1: 1094-1101.

Rossi FM, Blau HM (1998) Recent advances in inducible gene expression systems. Curr Opin Biotechnol 9: 451-456.

Rotzler S, Schramek H, Brenner HR (1991) Metabolic stabilization of endplate acetylcholine receptors regulated by Ca2+ influx associated with muscle activity. Nature 349: 337-339.

Rozakis-Adcock M, McGlade J, Mbamalu G, Pelicci G, Daly R, Li W, Batzer A, Thomas S, Brugge J, Pelicci PG (1992) Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. Nature 360: 689-692.

Rozakis-Adcock M, van der GP, Mbamalu G, Pawson T (1995) MAP kinase phosphorylation of mSos1 promotes dissociation of mSos1-Shc and mSos1-EGF receptor complexes. Oncogene 11: 1417-1426.

Ruegg MA, Bixby JL (1998) Agrin orchestrates synaptic differentiation at the vertebrate neuromuscular junction. Trends Neurosci 21: 22-27.

Ruegg MA, Tsim KW, Horton SE, Kroger S, Escher G, Gensch EM, McMahan UJ (1992) The agrin gene codes for a family of basal lamina proteins that differ in function and distribution. Neuron 8: 691-699.

Saher G, Hildt E (1999) Activation of c-raf-1 kinase signal transduction pathway in alpha(7) integrin-deficient mice. J Biol Chem 274:27651-27657.

Salpeter MM, Marchaterre M, Harris R (1988) Distribution of extrajunctional acetylcholine receptors on a vertebrate muscle: evaluated by using a scanning electron microscope autoradiographic procedure. J Cell Biol 106: 2087-2093.

Sandrock AW, Jr., Dryer SE, Rosen KM, Gozani SN, Kramer R, Theill LE, Fischbach GD (1997) Maintenance of acetylcholine receptor number by neuregulins at the neuromuscular junction in vivo. Science 276: 599-603.

Sanes JR (1982) Laminin, fibronectin, and collagen in synaptic and extrasynaptic portions of muscle fiber basement membrane. J Cell Biol 93: 442-451.

Sanes JR, Apel ED, Burgess RW, Emerson RB, Feng G, Gautam M, Glass D, Grady RM, Krejci E, Lichtman JW, Lu JT, Massoulie J, Miner JH, Moscoso LM, Nguyen Q, Nichol M, Noakes PG, Patton BL, Son YJ, Yancopoulos GD, Zhou H (1998) Development of the neuromuscular junction: genetic analysis in mice. J Physiol Paris 92: 167-172.

Sanes JR, Cheney JM (1982) Lectin binding reveals a synapse-specific carbohydrate in skeletal muscle. Nature 300: 646-647.

Sanes JR, Hall ZW (1979) Antibodies that bind specifically to synaptic sites on muscle fiber basal lamina. J Cell Biol 83: 357-370.

Sanes JR, Johnson YR, Kotzbauer PT, Mudd J, Hanley T, Martinou JC, Merlie JP (1991) Selective expression of an acetylcholine receptor-lacZ transgene in synaptic nuclei of adult muscle fibers. Development 113: 1181-1191.

Sanes JR, Lichtman JW (1999) Development of the vertebrate neuromuscular junction. Annu Rev Neurosci 22: 389-442.

Sanes JR, Marshall LM, McMahan UJ (1978) Reinnervation of muscle fiber basal lamina after removal of myofibers. Differentiation of regenerating axons at original synaptic sites. J Cell Biol 78: 176-198.

Sasaoka T, Draznin B, Leitner JW, Langlois WJ, Olefsky JM (1994) Shc is the predominant signaling molecule coupling insulin receptors to activation of guanine

nucleotide releasing factor and p21ras-GTP formation. J Biol Chem 269: 10734-10738.

Satoh T, Endo M, Nakafuku M, Akiyama T, Yamamoto T, Kaziro Y (1990) Accumulation of p21ras.GTP in response to stimulation with epidermal growth factor and oncogene products with tyrosine kinase activity. PNAS 87: 7926-7929.

Satoh T, Nakafuku M, Kaziro Y (1992) Function of Ras as a molecular switch in signal transduction. J Biol Chem 267: 24149-24152.

Scheele JS, Rhee JM, Boss GR (1992) Determination of absolute amounts of GDP and GTP bound to Ras in mammalian cells: comparison of parental and Ras-overproducing NIH 3T3 fibroblasts. PNAS 92: 1097-1100.

Schlaepfer DD, Jones KC, Hunter T (1998) Multiple Grb2-mediated integrin-stimulated signaling pathways to ERK2/mitogen-activated protein kinase: summation of both c-Src- and focal adhesion kinase-initiated tyrosine phosphorylation events. Mol Cell Biol 18: 2571-2585.

Schlessinger J (1993) How receptor tyrosine kinases activate Ras. Trends Biochem Sci 18: 273-275.

Schneider C, Newman RA, Sutherland DR, Asser U, Greaves MF (1982) A one-step purification of membrane proteins using a high efficiency immunomatrix. J Biol Chem 257: 10766-10769.

Scott LJ, Bacou F, Sanes JR (1988) A synapse-specific carbohydrate at the neuromuscular junction: association with both acetylcholinesterase and a glycolipid. J Neurosci 8: 932-944.

Seabra MC (1998) Membrane association and targeting of prenylated Ras-like GTPases. Cell Signal 10: 167-172.

Sealock R, Wray BE, Froehner SC (1984) Ultrastructural localization of the Mr 43,000 protein and the acetylcholine receptor in Torpedo postsynaptic membranes using monoclonal antibodies. J Cell Biol 98: 2239-2244.

Shadiack AM, Nitkin RM (1991) Agrin induces alpha-actinin, filamin, and vinculin to co-localize with AChR clusters on cultured chick myotubes. J Neurobiol 22: 617-628.

Si J, Luo Z, Mei L (1996) Induction of acetylcholine receptor gene expression by ARIA requires activation of mitogen-activated protein kinase. J Biol Chem 271: 19752-19759.

Si J, Mei L (1999) ERK MAP kinase activation is required for acetylcholine receptor inducing activity-induced increase in all five acetylcholine receptor subunit mRNAs as well as synapse-specific expression of acetylcholine receptor varepsilon-transgene. Brain Res Mol Brain Res 67: 18-27.

Simon MA, Dodson GS, Rubin GM (1993) An SH3-SH2-SH3 protein is required for p21Ras1 activation and binds to sevenless and Sos proteins in vitro. Cell 73: 169-177.

Slater CR (1990) The basal lamina and stability of the mammalian neuromuscular junction. Prog Brain Res 84: 73-81.

Smith MA, Fanger GR, O'Connor LT, Bridle P, Maue RA (1997) Selective regulation of agrin mRNA induction and alternative splicing in PC12 cells by Ras-dependent actions of nerve growth factor. J Biol Chem 272: 15675-15681.

Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, King F, Roberts T, Ratnofsky S, Lechleider RJ (1993) SH2 domains recognize specific phosphopeptide sequences. Cell 72: 767-778.

Steinbach JH (1981) Developmental changes in acetylcholine receptor aggregates at rat skeletal muscle neuromuscular junctions. Dev Biol 84: 267-276.

Stephens RM, Loeb DM, Copeland TD, Pawson T, Greene LA, Kaplan DR (1994) Trk receptors use redundant signal transduction pathways involving SHC and PLC-gamma 1 to mediate NGF responses. Neuron 12: 691-705.

Sternberg EA, Spizz G, Perry ME, Olson EN (1989) A ras-dependent pathway abolishes activity of a muscle-specific enhancer upstream from the muscle creatine kinase gene. Mol Cell Biol 9: 594-601.

Stevenson LE, Frackelton AR, Jr. (1998) Constitutively tyrosine phosphorylated p52 Shc in breast cancer cells: correlation with ErbB2 and p66 Shc expression. Breast Cancer Res Treat 49: 119-128.

Stevenson LE, Ravichandran KS, Frackelton AR, Jr. (1999) Shc dominant negative disrupts cell cycle progression in both G0-G1 and G2-M of ErbB2-positive breast cancer cells. Cell Growth Differ 10: 61-71.

Stokoe D, Macdonald SG, Cadwallader K, Symons M, Hancock JF (1994) Activation of Raf as a result of recruitment to the plasma membrane. Nature 264: 1463-1467.

Sturani E, Abbondio A, Branduardi P, Ferrari C, Zippel R, Martegani E, Vanoni M, Denis-Donini S (1997) The Ras Guanine nucleotide Exchange Factor CDC25Mm is present at the synaptic junction. Exp Cell Res 235 : 117-123.

Sugiyama J, Bowen DC, Hall ZW (1994) Dystroglycan binds nerve and muscle agrin. Neuron 13: 103-115.

Sugiyama JE, Glass DJ, Yancopoulos GD, Hall ZW (1997) Laminin-induced acetylcholine receptor clustering: an alternative pathway. J Cell Biol 139: 181-191.

Swope SL, Huganir RL (1993) Molecular cloning of two abundant protein tyrosine kinases in Torpedo electric organ that associate with the acetylcholine receptor. J Biol Chem 268: 25152-25161.

Swope SL, Huganir RL (1994) Binding of the nicotinic acetylcholine receptor to SH2 domains of Fyn and Fyk protein tyrosine kinases. J Biol Chem 269: 29817-29824.

Szeberenyi J, Cai H, Cooper GM (1990) Effect of a dominant inhibitory Ha-ras mutation on neuronal differentiation of PC12 cells. Mol Cell Biol 10: 5324-5332.

Takano H, Komuro I, Oka T, Shiojima I, Hiroi Y, Mizuno T, Yazaki Y (1998) The Rho family G proteins play a critical role in muscle differentiation. Mol Cell Biol 18: 1580-1589.

Tanowitz M, Si J, Yu DH, Feng GS, Mei L (1999) Regulation of neuregulin-mediated acetylcholine receptor synthesis by protein tyrosine phosphatase. J Neurosci 19: 9426-9435.

Tansey MG, Chu GC, Merlie JP (1996) ARIA/HRG regulates AChR epsilon subunit gene expression at the neuromuscular synapse via activation of phosphatidylinositol 3-kinase and Ras/MAPK pathway. J Cell Biol 134: 465-476.

Tsakiridis T, Bergman A, Somwar R, Taha C, Aktories K, Cruz TF, Klip A, Downey GP (1998) Actin filaments facilitate insulin activation of the src and collagen homologous/mitogen-activated protein kinase pathway leading to DNA synthesis and c-fos expression. J Biol Chem 273: 28322-28331.

Tsim KW, Ruegg MA, Escher G, Kroger S, McMahan UJ (1992) cDNA that encodes active agrin. Neuron 8: 677-689.

Vaidya TB, Weyman CM, Teegarden D, Ashendel CL, Taparowsky EJ (1991) Inhibition of myogenesis by the H-ras oncogene: implication of a role for protein kinase C. J Cell Biol 114: 809-820.

Valenzuela DM, Stitt TN, DiStefano PS, Rojas E, Mattsson K, Compton DL, Nunez L, Park JS, Stark JL, Gies DR (1995) Receptor tyrosine kinase specific for the skeletal

muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. Neuron 15: 573-584.

van der GP, Wiley S, Pawson T (1999) Re-engineering the target specificity of the insulin receptor by modification of a PTB domain binding site. Oncogene 18: 3071-3075.

VanderKuur J, Allevato G, Billestrup N, Norstedt G, Carter-Su C (1995) Growth hormone-promoted tyrosyl phosphorylation of SHC proteins and SHC association with Grb2. J Biol Chem 270: 7587-7593.

Vetter ML, Martin-Zanca D, Parada LF, Bishop JM, Kaplan DR (1991) Nerve growth factor rapidly stimulates tyrosine phosphorylation of phospholipase C-gamma 1 by a kinase activity associated with the product of the trk protooncogene. Proc Natl Acad Sci U S A 88: 5650-5654.

Vijapurkar U, Cheng K, Koland JG (1998) Mutation of a Shc binding site tyrosine residue in ErbB3/HER3 blocks heregulin-dependent activation of mitogen-activated protein kinase. J Biol Chem 273: 20996-21002.

Vojtek AB, Der CJ (1998) Increasing complexity of the Ras signaling pathway. J Biol Chem 273: 19925-19928.

Wallace BG (1988) Regulation of agrin-induced acetylcholine receptor aggregation by Ca++ and phorbol ester. J Cell Biol 107: 267-278.

Wallace BG (1990) Inhibition of agrin-induced acetylcholine-receptor aggregation by heparin, heparan sulfate, and other polyanions. J Neurosci 10: 3576-3582.

Wallace BG (1994) Staurosporine inhibits agrin-induced acetylcholine receptor phosphorylation and aggregation. J Cell Biol 125: 661-668.

Wallace BG (1995) Regulation of the interaction of nicotinic acetylcholine receptors with the cytoskeleton by agrin-activated protein tyrosine kinase. J Cell Biol 128: 1121-1129.

Wallace BG (1996) Signaling mechanisms mediating synapse formation. Bioessays 18: 777-780.

Wallace BG, Qu Z, Huganir RL (1991) Agrin induces phosphorylation of the nicotinic acetylcholine receptor. Neuron 6: 869-878.

Wang S, Ghosh RN, Chellappan SP (1998) Raf-1 physically interacts with Rb and regulates its function: a link between mitogenic signaling and cell cycle regulation. Mol Cell Biol 18:7487-7498.

Waters SB, Holt KH, Ross SE, Syu LJ, Guan KL, Saltiel AR, Koretzky GA, Pessin JE (1995a) Desensitization of Ras activation by a feedback disassociation of the SOS-Grb2 complex. J Biol Chem 270: 20883-20886.

Waters SB, Yamauchi K, Pessin JE (1995b) Insulin-stimulated disassociation of the SOS-Grb2 complex. Mol Cell Biol 15: 2791-2799.

Watson FL, Porcionatto MA, Bhattacharyya A, Stiles CD, Segal RA (1999) TrkA glycosylation regulates receptor localization and activity. J Neurobiol 39: 323-336.

Weed SA, Du Y, Parsons JT (1998) Translocation of cortactin to the cell periphery is mediated by the small GTPase Rac1. J Cell Sci 111 (Pt 16): 2433-2443.

Wei L, Zhou W, Croissant JD, Johansen FE, Prywes R, Balasubramanyam A, Schwartz RJ (1998) RhoA signaling via serum response factor plays an obligatory role in myogenic differentiation. J Biol Chem 273: 30287-30294.

Wells DG, Fallon JR (1996) The state of the union. Neuromuscular junction. Curr Biol 6: 1073-1075.

Wells DG, McKechnie BA, Kelkar S, Fallon JR (1999) Neurotrophins regulate agrin-induced postsynaptic differentiation. Proc Natl Acad Sci U S A 96: 1112-1117.

Weyman CM, Ramocki MB, Taparowsky EJ, Wolfman A (1997) Distinct signaling pathways regulate transformation and inhibition of skeletal muscle differentiation by oncogenic Ras. Oncogene 14: 697-704.

Weyman CM, Wolfman A (1997) Oncogenic Ras-induced secretion of a novel inhibitor of skeletal myoblast differentiation. Oncogene 15: 2521-2528.

White MA, Nicolette C, Minden A, Polverino A, Van Aelst L, Karin M, Wigler MH (1995) Multiple Ras functions can contribute to mammalian cell transformation. Cell 80: 533-541.

Wittinghofer A, Herrmann C (1995) Ras-effector interactions, the problem of specificity. FEBS Lett 369: 52-56.

Wu H, Montone KT (1998) Cortactin localization in actin-containing adult and fetal tissues. J Histochem Cytochem 46: 1189-1191.

Wu H, Parsons JT (1993) Cortactin, an 80/85-kilodalton pp60src substrate, is a filamentous actin-binding protein enriched in the cell cortex. J Cell Biol 120: 1417-1426.

Wu H, Reynolds AB, Kanner SB, Vines RR, Parsons JT (1991) Identification and characterization of a novel cytoskeleton-associated pp60src substrate. Mol Cell Biol 11: 5113-5124.

Xia K, Lee RS, Narsimhan RP, Mukhopadhyay NK, Neel BG, Roberts TM (1999) Tyrosine phosphorylation of the proto-oncoprotein Raf-1 is regulated by Raf-1 itself and the phosphatase Cdc25A. Mol Cell Biol 19: 4819-4824.

Yang B, Jung D, Motto D, Meyer J, Koretzky G, Campbell KP (1995) SH3 domain-mediated interaction of dystroglycan and Grb2. J Biol Chem 270: 11711-11714.

Yeh TC, Ogawa W, Danielsen AG, Roth RA (1996) Characterization and cloning of a 58/53-kDa substrate of the insulin receptor tyrosine kinase. J Biol Chem 271: 2921-2928.

Yoon SO, Soltoff SP, Chao MV (1997) A dominant role of the juxtamembrane region of the TrkA nerve growth factor receptor during neuronal cell differentiation. J Biol Chem 272: 23231-23238.

York RD, Yao H, Dillon T, Ellig CL, Eckert SP, McCleskey EW, Stork PJ (1998) Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. Nature 392: 622-626.

Zhang JM, Wei Q, Zhao X, Paterson BM (1999) Coupling of the cell cycle and myogenesis through the cyclin D1- dependent interaction of MyoD with cdk4. EMBO J 18: 926-933.

Zhou H, Glass DJ, Yancopoulos GD, Sanes JR (1999) Distinct domains of MuSK mediate its abilities to induce and to associate with postsynaptic specializations. J Cell Biol 146: 1133-1146.

Zhou MM, Harlan JE, Wade WS, Crosby S, Ravichandran KS, Burakoff SJ, Fesik SW (1995) Binding affinities of tyrosine-phosphorylated peptides to the COOH- terminal SH2 and NH2-terminal phosphotyrosine binding domains of Shc. J Biol Chem 270: 31119-31123.

Zhu X, Lai C, Thomas S, Burden SJ (1995) Neuregulin receptors, erbB3 and erbB4, are localized at neuromuscular synapses. EMBO J 14: 5842-5848.