Molecular Phenotype of Fragile X Syndrome: FMRP, FXRPs, and Protein Targets

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ABSTRACT Fragile X syndrome (FraX) is one of the most prevalent genetic causes of mental retardation. FraX is associated with an unstable expansion of a polymorphism within the 5′ untranslated region of the FMR1 gene. The main consequence of this mutation is a reduction in the levels of the gene product (FMRP). FMRP is an RNA-binding protein with multiple spliced variants (isoforms) and high levels of expression in a variety of tissues, including neurons. In the latter cells, it is localized not only to the perikaryon but also to dendrites and dendritic spines. FMRP belongs to a family of proteins that includes the Fragile X Related Proteins or FXRPs. FXRPs share high homology in their functional domains with FMRP, and also associate with mRNA and components of the protein synthesis apparatus. However, FXRPs do not have the same temporo-spatial pattern of distribution (and other properties) of FMRP. Immunohistochemical assays have confirmed that a functionally uncompensated FMRP deficit is the essence of the FraX molecular phenotype. Here, we report our preliminary study on FXRPs levels in leukocytes from FraX males. By immunoblotting, we found that a marked reduction in FMRP levels is associated with a modest increase in FXR1P and no changes in FXR2P levels. The consequences of this reduced FMRP expression on protein synthesis, in other words, the identification of FXRPs targets, can be studied by different molecular approaches including protein interaction and proteomics methods. By two-dimensional gel electrophoresis, we showed that in FraX leukocytes there is a defect in acetylation that involves prominently the regulatory protein annexin-1. Extension of current studies of the molecular phenotype to more brain-relevant tissue samples, a wider range of proteomics-based methods, and correlative analyses of FXRPs homologues and FMRP targets with multiple behavioral measures, will greatly expand our understanding of FraX pathogenesis and it will help to develop and monitor new therapeutic strategies. Microsc. Res. Tech. 57:135–144, 2002. © 2002 Wiley-Liss, Inc.

INTRODUCTION

Fragile X syndrome (FraX) is one of the most prevalent genetic causes of mental retardation, representing the most frequent form of inherited severe cognitive deficit, second only to Down syndrome (Kaufmann and Moser, 2000). In a majority of cases, the mutation consists of an unstable expansion of a CGG trinucleotide repeat within the 5′ untranslated region (UTR) of the FMR1 gene. Based on the size of this expansion, alleles are classified as normal (5–40 repeats), intermediate or gray zone (41–60 repeats), premutation (PM, 61–199 repeats), or full mutation (FM, 200–2,000 repeats) alleles (Kaufmann and Reiss, 1999; Nolin et al., 1996). A mixed pattern of FMR1 mutation in which PM and FM alleles are combined is termed mosaicism (Mos) (Oberlé et al., 1991; Rousseau et al., 1994, 1995; Verkerk et al., 1991). Only FM-size repeat expansions are regularly associated with hypermethylation of both the CGG polymorphic region and a CpG island located 250 bp proximal to it, which leads to gene silencing and a severe physical and neurologic phenotype (Kaufmann and Reiss, 1999; Oostra and Halley, 1995). This contrasts with typical and methylation mosaics (i.e., unmethylated FM alleles) that show a generally milder cognitive impairment (Hagerman et al., 1994; Kaufmann et al., 1999). These data suggest that the most important factor determining FMR1 expression and FraX phenotype is the degree of FMR1 methylation, and, therefore, gene silencing, not necessarily the length of the FMR1 expansion (Feng et al., 1995; Hagerman et al., 1994). For this reason, any description of the FraX neurobehavioral phenotype requires an initial characterization of FraX’s molecular phenotype: the levels and pattern of expression of the FMR1 gene product, the Fragile X Mental Retardation Protein (FMRP).

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imens of males with FM. MW standards are in kilodaltons. Reproduced from Kaufmann et al. (1999) with permission of the publisher.

FMR1 normal and (most of) PM alleles are transcribed, their pre-mRNA alternatively spliced, and translated into several FMR isoforms (Ashley et al., 1993; Pieretti et al., 1991). These FMR isoforms, which differ mainly in their C-terminus (see Fig. 1), are the consequence of FMR1 mRNA splice variants. Initial studies that focused on lymphoblastoid cell lines demonstrated that these cells expressed at least four different FMRP isoforms (Devys et al. 1993; Verheij et al., 1995). FMRP isoforms range in molecular weight between 67 and 80 kD, with relatively more abundance of the latter isoforms (Verheij et al., 1993, 1995). However, kidney and liver FMRP isoforms from human, monkey, and mouse appear to have lower molecular weights of ~40 kD. These lighter FMRPs, which share their amino (N)-terminal region with the 67–80-kD forms, are most likely the consequence of C-terminus cleavage since the smallest FMRP isoform predicted by mRNA analyses is ~45 kD.

Although FMRP isoforms are expressed similarly in many tissues and organs, the relative abundance of FMRP is tissue-specific. Immunoblotting and immunocytochemical studies indicate that FMRP levels are highest in brain, testis, and epithelia. Mesodermal- and endodermal-origin tissues, such as muscle, liver, and mammary gland, have lower FMRP expression (Devys et al., 1993; Khandjian et al., 1995). Within each tissue, specific cell-type FMRP distribution has been noticed. In brain, FMRP is predominantly neuronal, while in testis spermatogonia but not more mature germ or Sertoli cells are immunolabeled (Devys et al., 1993). FMRP is predominantly cytoplasmic, but it also localizes to the nucleus of several cell types (Devys et al., 1993; Feng et al., 1997b; Verheij et al., 1993). Accordingly, recent molecular studies have confirmed both nuclear localization and nuclear export signals within FMRP (Eberhart et al., 1996; Fridell et al., 1996). Feng and colleagues (1997b) have expanded initial observations in humans (Devys et al., 1993), and demonstrated that in neurons FMRP is localized not only in the perikaryal region but also in dendrites and dendritic spines (Figs. 2 and 3).

An initial insight into FMRP function was obtained from analyses of atypical FMR1 mutations. These unusual cases that do show a relatively typical FraX phenotype do not have FMR1 CGG expansions, but a different type of deletions and single point mutations (De Bouelle et al., 1993; Gedeon et al., 1992; Hirst et al., 1995; Meijer et al., 1994; Wohlrle et al., 1992). The latter mutation, which consisted in an A→T substitution that led to Ile→Asn change in residue 367, emphasized the importance of some FMRP domains. FMRP sequence shows similarities with other RNA-binding proteins (Ashley et al., 1993; Sioni et al., 1993); three domains involved in RNA binding are found in FMRP: two heterogeneous nuclear ribonucleoprotein K homology (KH) domains in the middle of the molecule (222–258 and 284–320, respectively) and one RGG box near the C-terminus (residues 527–552) (Fig. 2).
Fig. 3. Electron micrograph demonstrating FMRP localization in cellular processes in cerebral cortex. A–C: Dendrites (d) in cross-section and longitudinal section showing that immunogold particles are either free in the cytoplasm or clustered around cisternae of smooth ER (arrows) or at the origins of dendritic spines (arrowheads). D–F: Dendritic spines (s) containing immunogold particles, which are either free in the cytoplasm or associated with the spine apparatus (arrows). G: Rare axon terminals (a) contain immunogold particles that are cytoplasmic in location. H: FMRP-immunoreactive axon terminals (a) are more easily identified using immunoperoxidase. Scale bars: A–H, 500 nm. Reproduced from Feng et al. (1997) with permission of the publisher.

4; Verkerk et al., 1993). The Ile367 substitution observed in the patient mentioned above, which is considered to be localized to the second KH domain, reduced RNA binding in vitro (Siomi et al., 1994) despite appropriate FMRP synthesis and intracellular localization (Verheij et al., 1995). As this case demonstrates the importance of the KH domains in FMRP RNA-binding properties, absence of the RGG box also leads to FMRP inability to bind RNA (Siomi et al., 1993). Hence, FMRP isoforms lacking carboxy-terminal sequences should have impairment in RNA binding. Indeed, Verheij et al. (1995) demonstrated that the ~40-kd FMRPs found in visceral organs do not bind to RNA homopolymers (Verheij et al., 1995). FMRP RNA binding has been demonstrated by in vitro (Siomi et al., 1994) and in vivo assays (Verheij et al., 1995). In vitro translated FMRP interacts with its own mRNA, a subset of brain transcripts, and DNA (Ashley et al., 1993). These authors estimated that FMRP binds to approximately 4% fetal brain mRNA in a 1:2 ratio (2 RNA molecules per FMRP) (Ashley et al., 1993). Tissue fractionation assays and electron microscopic studies have shown that FMRP co-localizes with or binds to rough endoplasmic reticulum (RER)-associated ribosomes, free polysomes, and nucleolar granular component (Eberhart et al., 1996; Feng et al., 1997a; Khandjian et al., 1996; Siomi et al., 1996; Willemsen et al., 1996). Furthermore, FMRP isoforms are associated as cytoplasmic mRNPs with actively translating polyribosomes (Corbin et al., 1997; Feng et al., 1997a; Tamanini et al., 1999b). The association of FMRP with the ribosomal 60S subunit appears to occur via RNA binding (Eberhart et al., 1996; Tamanini et al., 1996). Recent data indicate that FMRP’s role in protein synthesis is mainly inhibitory, via direct interaction with mRNA and other messenger ribonucleoproteins (mRNPs; Li et al., 2001). This translation inhibitory function appears to be specific to FMRP, since FMRP homologues fail to suppress translation at similar concentration ranges, and involves RNA-binding domains since it is absent in the Ile367 substitution described above (Laggerbauer et al., 2001).

Fig. 4. Schematic representation of FMRP. This diagram corresponds to the polypeptide encoded by the largest FMR1 transcript. The three RNA-binding domains are represented as open boxes. The two KH domains are in the midportion of FMRP, comprising residues 222–258 and 284–320, whereas the RGG box spans amino acids 527–552. The monoclonal antibody (mAb) 1a, which has been used in our studies (see Fig. 3), recognizes an epitope in the N-terminus portion of FMRP as indicated by the underlying line. Adapted from Verheij et al. (1995).

**FMRP HOMOLOGUES: THE FRAGILE X RELATED PROTEINS (FXRPs)**

FMRP is a member of a family of RNA-binding proteins, which includes at least two proteins coded for by autosomal genes (Siomi et al., 1995; Zhang et al., 1995). FXR1P and FXR2P have a similar overall structure to FMRP (~60% amino acid identity) (Tamanini et al., 1997). In the case of FXR1P, there is 70% amino acid identity with FMRP in the N-terminal region and 86% in the central region of the molecule (Khandjian et al., 1998; Siomi et al., 1995, 1996; Verheij et al., 1995). FXR1P and FXR2P also have two KH domains and one RGG box, as well as also bind RNA homopolymers and are associated, as cytoplasmic mRNPs, to the ribosomal 60S subunit (Siomi et al., 1996; Tamanini et al., 1996, 1999b; Zhang et al., 1995). FMRP, FXR1P, and FXR2P are able to bind their own transcripts and to each other, although these homo- and heterodimers are of unknown significance. In the human adult brain, the three proteins co-localize in the perikarya of several neuronal populations including cortical cells. By contrast, during the fetal period FMRP and FXR2P are cytoplasmic while FXR1P is predominantly nuclear (Tamanini et al., 1997). These authors also demonstrated lack of co-localization in adult and fetal testis (Tamanini et al., 1997). Tamanini et al. (1999a) have recently shown that FMRP and FXR2P may have different transcript targets since their nucleocytoplasmic shuttling patterns are distinct. Of the two FMRP homologues, FXR1P is better known. Khandjian and colleagues (1998) recently showed that in addition to the short (70 kd) and long (78 kd) FXR1P isoforms initially described (Siomi et al., 1995), there are higher molecular weight (HMW) isoforms, 81–84 kd, that replace the 70- and 78-kd isoforms in muscle after these cells are induced to differentiate. As in the case of FMRP, these novel variants that have variable polyribosomal association are originated by alternative splicing (Khandjian et al., 1998). Khandjian et al. (1998) also demonstrated that in normal human lymphoblasts, there is a higher relative expression of FMRP and 70-kd FXR1P than 78-kd FXR1P and FXR2P. Moreover, Khandjian et al. (1998), using an FMRP antibody (Ab) that targets the highly homologous N-terminal region, found two weak 70- and 78-kd bands in brain homogenates from the FMR1 knockout mouse, which...
they interpreted as a cross-reaction with FXR1P. These data support the existence of a complex system of FMRP-like molecules that appear to regulate protein synthesis in neural and non-neural tissues. The relative capacity of this system of proteins for compensating FMRP deficiency in FraX is still unknown; several possibilities are discussed in a following section. A complementary review about FXRPs is included in the paper by Hoogeveen and colleagues (pages 148–155, this issue).

MOLECULAR PHENOTYPE IN FRAGILE X: MEASURING FMRP EXPRESSION

Most studies aiming to determine the levels and pattern of FMRP expression in FraX have focused on peripheral blood samples. Following initial immunocytochemical evaluations of lymphoblastoid cell lines and postmortem tissue samples (Devys et al., 1993; Feng et al., 1995; Verheij et al., 1993, 1995), several assays for assessing FMRP expression in non-transformed lymphocytes have been developed. The advantages of studying these fresh blood samples include their simpler features (that allow faster assays) and the fact that non-transformed cells are more representative of the diverse cell populations found in one individual. For the latter reason, lymphocyte-based assays have become the most suitable FMRP measure for molecular-behavioral correlations (Kaufmann et al., 1999; Tassone et al., 1999). Immunocytochemical assays for measuring FMRP expression in peripheral leukocytes have been reported by Willemsen et al. (1995) and Tassone and colleagues (1999). More recently, a hair root-based immunocytochemical protocol, of great potential for epidemiologic studies (Tuncbilek et al., 2000), has been described by Willemsen and collaborators (1999). Although the value of these immunocytochemical assays, in terms of feasibility and accuracy, has been clearly demonstrated, they lack a true quantitative nature. Moreover, immunocytochemical techniques are not able to characterize the molecular components contributing to the detected immunoreactivity. Consequently, we implemented a lymphocyte homogenate-based immunoblotting assay to examine the molecular profiles representing the spectrum of FMR1 mutations (Fig. 1; Kaufmann et al., 1999). We confirmed a relatively normal FMRP expression in subjects with PM and in a large proportion of females with PM, while FMRP levels in males with PM and mosaicism were markedly reduced (Kaufmann et al., 1999). Nevertheless, recent studies with more sensitive molecular techniques have shown that males with large PM alleles (100–200 repeats), or with unmethylated PM alleles (methylation Mos), have reduced FMRP levels despite significantly elevated FMR1 mRNA levels (Kenneson et al., 2001; Tassone et al., 2000a). These results highlight the significance of developing sensitive immunochromic methods, not yet available for routine evaluations, for determining FMRP expression. The critical importance of accurate molecular-behavioral correlations is further emphasized by reports indicating that a significant proportion of females with PM may have premature ovarian failure (Sherman, 2000) and that older males with PM can develop a progressive neurologic disorder (Hagerman et al., 2001).

FRAGILE X MOLECULAR PHENOTYPE: UNCOMPENSATED FMRP DEFICIT

Our data, as well as that of other groups, have demonstrated that only males with PM and mosaicism show a marked reduction in FMRP expression (Fig. 1). In these individuals, any FMRP immunoreactivity should correspond to the relatively most abundant 80-kd FMRP (Kaufmann et al., 1999; Verheij et al., 1993, 1995). Nevertheless, using an Ab that recognizes an epitope comprising residues 1–150 of FMRP, we detected an ~70-kd band that was invariably present despite the absence of higher molecular weight bands (Fig. 1, lane 3). This 70-kd FMRP-like immunoreactivity was also distinguished in males with Mos and in females with low-levels of FMRP expression (Fig. 1, lane 2; Kaufmann et al., 1999). We interpret these results as the presence of an FMRP-like protein, probably compensatory in nature. Supporting this hypothesis is the report by Khandjian et al. colleagues (1999) of weak 70- and 78-kd bands in brains of the FMR1 knockout mice. Our 1a Ab, as Khandjian’s 1C3 (Khandjian et al., 1998), targets the same highly homologous N-terminal region of the FMRP/FXRPs family of proteins (Zhang et al., 1995). Considering these data, and the finding that in normal lymphoblasts the most abundantly expressed members of this family of proteins are FMRP and 70-kd FXR1P (Khandjian et al., 1998), we postulate that the 70-kd FMRP-like immunoreactivity in males with PM and mosaicism is, indeed, 70-kd FXR1P.

In order to determine whether the FraX phenotype is due to an incapacity for compensating FMRP by the FXRPs, we performed a preliminary quantitative immunoblotting study of 78-kd FXR1P and FXR2P in leukocyte homogenates from males with different levels of FMRP deficit. On the bases of FMRP levels, individuals were categorized as low and high FMRP expressing. The former group included 13 males with FM and low-level mosaicism. The high FMRP level group consisted of 5 control and PM subjects. As Figure 5 shows, we found a moderate, although not significant, elevation only in FXR1P. FXR2P levels were comparable in both groups of males. These data altogether suggest that, in peripheral leukocytes, there is moderate elevation in 70- and 78-kd FXR1P, in response to reduced FMRP levels. The lack of a functional consequence of this FXRP “compensatory” response may be explained by its modest magnitude, its circumscription to FXR1P, and also possibly by its limited tissue distribution. Although at present there are no data on FMRP/FXRPs expression in FraX neural tissues, the fact that all three proteins are co-expressed in a wide variety of neuronal populations (Tamanini et al., 1997) suggests that if, as in leukocytes, a modest elevation in FXR1P is associated with severe FMRP deficit, this response is largely ineffective. While FMRP’s postulated role in synaptic activity in the adult brain (Greenough et al., 2001) might explain some elements of the FraX phenotype, additional features of this system of proteins provide a better understanding of the lack of FMRP compensation during development that could result in a dysfunctional brain. During fetal life, in neurons FMRP and FXR2P are mainly cytoplasmic while FXR1P is predominantly nuclear (Tamanini et
al., 1997). Therefore, a reduction in FMRP could not be counterbalanced by the most abundant FXR1P since this protein is in a different neuronal compartment. In this way, only FXR2P has the potential of offsetting this protein is in a different neuronal compartment. In counterbalanced by the most abundant FXR1P since al., 1997). Therefore, a reduction in FMRP could not be

**FMRP EXPRESSION IN BRAIN-RELEVANT TISSUES**

To date no studies have characterized FMRP or FXRPs expression in brain or other neural tissues from FraX patients. The limited availability of tissues suitable for molecular and immunobehavioral studies has emphasized evaluating animal models and the search for alternative tissue samples. The subject of animal models, relevant to FraX, is reviewed in detail by Hoogeveen et al. (pages 148–155, this issue) and Churchill et al. (pages 156–158, this issue). Here, we summarize our initial efforts of characterizing tissues functionally or embryologically related to brain. In order to determine whether FMRP expression in leukocytes is representative of FMR1 expression in the brain, we carried out a preliminary study of olfactory neuroblasts (ON) from two males with FM and two normal controls (Abrams et al., 1999). ON are neuron-like cells that constitute the receptor elements of the olfactory system in the nasal mucosa. ON were obtained through nasal biopsies, by isolation from other cells of the nasal mucosa. ON are obtained through nasal biopsies, by isolation from other cells of the nasal mucosa, and cultured under appropriate conditions (Wolozi et al., 1992, 1993). In this study, we found a high correspondence in terms of both FMR1 patterns and FMRP expression, as determined by Southern and Western blotting, respectively, between leukocytes and ON in controls and FraX affected individuals (Fig. 6).

Hair roots/follicles (HF) have recently been used to evaluate FMRP expression in a less invasive way (Tuncbilek et al., 2000; Willemsen et al., 1999). Because of their common ectodermic origin (Fuchs, 1998), HF components, such as the outer sheath, share several key proteins with the central nervous system. These include, in addition to FMRP, transcription factors involved in neuronal differentiation (e.g., JAK/STAT), signal transduction proteins (e.g., inositol trisphosphate receptor or IP3R), neurotrophic receptors (e.g., p75NTR), and cell adhesion molecules (e.g., NCAM). As depicted in Figure 7, we have developed protocols that allow complex molecular analyses of proteins involved in HF and neuronal development and function. In addition to detecting FMRP, we have successfully characterized in control subjects the low-affinity neurotrophic receptor, also termed p75NTR. This receptor is involved in both HF morphogenesis (Botchkareva et al., 1999) and neuronal differentiation and cell death (Wiesmann and de Vos, 2001). These novel molecular approaches will provide not only a better description of FMRP expression in brain-relevant tissue samples, but also of molecules that could be affected by FMRP deficit.

**FMRP TARGETS: EARLY APPLICATION OF PROTEOMICS TO FRAGILE X**

FMRP and its homologues FXRPs appear to regulate protein synthesis via direct binding to RNA or to different components of the protein synthesis apparatus (Eberhart et al., 1996; Feng et al., 1997a,b; Tamanini et al., 1996, 1999b). Based on this, intense efforts have recently been made to identify the specific transcript targets of FMRP. Different approaches have been used; they include: FMRP-RNA binding assays (Ceman et al., 1999; Sung et al., 2000), RNA differential display methods (Zhong et al., 1999), protein interaction assays (Bardoni et al., 1999; Schenck et al., 2001), and FMRP localization, trafficking, and interaction analyses (Beaulieu, 2000; Tamanini et al., 1999a,b). Although these studies have already found putative RNA targets encoding both known and novel proteins, identification of proteins that can link FMRP more directly with the physical and neurologic FraX phenotype has been less successful. This may be because, to the best of our knowledge, protein profiling (i.e., proteomics) techniques have not been systematically used in FraX research. Our recent application of two-dimensional gel electrophoresis (2D PAGE) to the search for potential FMRP targets has shown that, in leukocytes, annexin-1 (Anx-1) and a group of at least other 10 proteins are abnormally expressed in males with FraX (Sun et al., 2001). As illustrated in Figure 8, the main abnormality consists of the presence of multiple (rather than 1–2) spots on 2D PAGE, which represent Anx-1 forms with a higher iso-electric point (pI) but approximately the same molecular weight (~35 kd) (Sun et al., 2001). The presence and severity of the Anx-1 pattern are directly related to the FMRP deficit in leukocytes (Fig. 9) and, based on appearance, are most likely the consequence of abnormal acetylation of this protein. The specificity to FraX of this phenomenon was demonstrated by comparing leukocyte 2D PAGE patterns not only with controls, but also with individuals with Rett or Down syndromes.

Anx-1’s and other (yet to be determined) proteins’ 2D PAGE pattern, with increasing pI but relatively pre-
served molecular weight, suggests an increase in acetylated residues (i.e., hyperacetylation). Acetylation of lysine residues is a type of posttranslational protein modification that has only recently received attention in the literature (Kouzarides, 2000). In addition to histones, other nuclear proteins such as transcriptional regulators (e.g., p53) as well as non-nuclear proteins are also acetylated. This process involves relatively non-specific acetylases, such as histone acetyl-transferases, and several families of deacetylases (e.g., HDAC1-3 family). In contrast to phosphorylation, acetylation is usually part of a cascade of signal transduction that may involve initial events linked to phosphorylation (Kouzarides, 2000; Swank and Sweatt, 2001). Among the best-known non-nuclear acetylated proteins is \(H\)-tubulin, a protein that undergoes acetylation and detyrosination prior to polymerization into microtubules (Black et al., 1986; Kaufmann et al., 2000). Most likely, aberrant acetylation in FraX is a secondary phenomenon to changes in signaling pathways (e.g., kinase activity) due to deficit in FMRP.

Anx-1, also termed lipocortin 1, is a calcium- and phospholipid-binding protein that modulates inflammatory responses. It is expressed in a variety of cells, being more abundant in monocytes and microglia (Miele et al., 1988; Shaw et al., 1999). Among other functions, Anx-1 is a mediator of the inhibitory function exerted by glucocorticoids on the hypothalamic-pituitary-adrenal axis (Jessop, 1999). Interestingly, recent data show that cortisol levels, particularly after stress, are higher in males with FraX (Wisbeck et al., 2000). Consequently, we hypothesize that abnormal Anx-1 expression (secondary to FMRP deficit) underlies adrenal dysfunction in FraX. As cortisol abnormalities have been postulated to underlie social anxiety and avoidance in FraX, Anx-1 abnormalities may be responsible for some of these behavioral features of the FraX phenotype. Another important observation regarding Anx-1 is that its expression is selectively induced by exogenous glucocorticoids in brain regions (e.g., hippocampus) (Philip et al., 1997; Voermans et al., 1997) found to be involved in FraX (Kates et al., 1997; Kaufmann and Reiss, 1999).

Based on these preliminary 2D PAGE data (Sun et al., 2001), we propose that in FraX there is a generalized defect in acetylation that leads to, among other proteins, Anx-1 hyperacetylation. This would, in turn, alter the functional capacity of Anx-1 and other hyperacetylated proteins. For instance, the interaction between Anx-1 and its target proteins (e.g., S100C) is highly dependent on the acetylation status of its N-terminus (Hall et al., 1993; Lew-Wei-Bentley et al., 2000; Retey et al., 2000). Hyperacetylation of additional Anx-1’s residues (i.e., lysine) in FraX would lead to confor-
mational changes that would result in deficient binding of this protein to its targets in multiple tissues, including the brain. The relevance of abnormal acetylation to FraX is underscored by recent data showing that neuronal acetylation in limbic regions, postulated as involved in FraX (see paper by Kates and colleagues, pages 159–167, this issue), appears to be an important process mediating signal transduction and learning (Swank and Sweatt, 2001). Consequently, we hypothesize that hyperacetylation may be a mechanism that can lead to both structural (e.g., dendritic spine abnormalities) and functional (e.g., increased cortisol levels) aspects of the FraX phenotype.

In conclusion, proteomics-based approaches that target the effects of FMRP deficit on protein synthesis and posttranslational modifications, as those detected by 2D PAGE, appear to be promising. Expansion of these studies to other tissue samples, including those more relevant to neural function (see above), as well as incorporation of other techniques (i.e., protein chips) have the potential of identifying more primary and secondary targets of FMRP, some of which could be modified pharmacologically.

MOLECULAR-BEHAVIORAL CORRELATIONS IN FRAGILE X

Prior to FMRP studies, genotype-phenotype correlations were established by using variables such as activation ratio, a measure of potentially transcribable FMR1 (Reiss et al., 1995). With the availability of FMRP assays, we and other groups have begun to examine the impact of molecular changes in FraX upon its neurologic phenotype. These molecular phenotype-neurobehavioral phenotype correlative analyses have mainly been circumscribed to leukocyte-based measurements and basic cognitive and behavioral vari-
ables. Examples of these correlations include the high predictive value of FMRP expression for full scale IQ (FSIQ) variance, particularly when analyses are restricted to subjects in the intermediate range of FMR1 mutations (males with typical and methylation Mos, females with FM) (Kaufmann et al., 1999; Tassone et al., 1999). Tassone and collaborators (2000b) have also reported the association of mild to moderate reduction in FMRP levels in (mainly male) subjects with PM with cognitive impairment, as measured by IQ. One of the few examples of analyses of non-cognitive behavioral features is the recent report by Bailey and colleagues (2001) of a lack of association between FMRP expression and autistic behavior, as measured by the Childhood Autism Rating Scale, in boys with FraX.

A different type of molecular-behavioral correlation is the one reported by us on the relationship between different types of FMRP immunoreactivity (by immunoblotting) and FSIQ. We compared the predictive value for FSIQ of total FMRP (including the 70-kd FMRP-like band mentioned in the previous section on FMRP deficit), the most abundant 80-kd FMRP isoform, and the 70-kd FMRP-like immunoreactivity in a group of FraX males covering a wide spectrum of FMR1 mutations (16 FM, 8 Mos). We found that total FMRP immunoreactivity (not shown) and, in particular, HMW (80 kd) FMRP were highly correlated with IQ. In contrast, the 70-kd FMRP-like immunoreactivity did not predict IQ scores (Fig. 10). The association between 80-kd FMRP and FSIQ was at even greater levels of significance than those found in our previous study in a mixed male/female sample, in which total FMRP immunoreactivity was correlated with FSIQ variance (Kaufmann et al., 1999). The latter data suggest that characteristics of the FMRP/FXRPs molecule, such as length and, therefore, potentially RNA-binding capacity (Verheij et al., 1995), may be more important than quantity (levels) in functional terms. Molecular-behavioral analyses of other molecules involved in FraX pathogenesis, including FXRPs and FMRP targets, have not yet been performed in a systematic manner. The combination of the latter analyses and FMRP-behavioral correlations, involving more brain-relevant tissue samples (e.g., olfactory neuroblasts), will provide a better understanding of the impact of FMRP deficit on the FraX neurobehavioral phenotype. They will also constitute an accurate way to monitor the effect of future gene or replacement therapies.

CONCLUSIONS

The main consequence of mutations affecting the FMR1 gene is a reduction in the levels of the gene product, FMRP. This functionally uncompensated deficit in FMRP constitutes the FraX molecular phenotype. The inability of the FMRP homologues, termed FXRPs, for compensating FMRP deficiency may be due to multiple factors that, among others, include differential tissue localization, distinct tempo-spatial ontogenetic patterns, and differences in functional domains. Deficit in FMRP results in abnormal protein synthesis, which can be studied by different molecular approaches. One of the most direct strategies is the use of proteomics; its application in the form of 2D PAGE to FraX leukocytes reveals a defect of acetylation that involves prominently the regulatory protein annexin-1. Extension of current studies of the molecular phenotype to more brain-relevant tissue samples, a wider range of proteomics-based methods, and correlative analyses of FMRP homologues and FMRP targets with multiple behavioral measures, will greatly expand our understanding of FraX pathogenesis. It will also help to develop and monitor new therapeutic strategies.

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